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Dkt. 41426-D/JPW/CY

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Ron S. Israeli et al.

Serial No.: 08/470,735 Group Art Unit: 1647

Filed: June 6, 1995 Examiner: S. Gucker

For: PROSTATE-SPECIFIC MEMBRANE ANTIGEN

1185 Avenue of the Americas
New York, New York 10036
June 16, 2004

Date of Notice Of Allowance: April 20, 2004 **Confirmation No.: 7764**

VIA HAND
U.S. Patent and Trademark Office
220 20th Street S.
Customer Window, Mail Stop Petition
Crystal Plaza Two, Lobby, Room 1B03
Arlington, VA 22202

ATTN: Director of Technology Center 1600

Sir:

PETITION TO WITHDRAW FROM ISSUE AT THE INTITIATIVE OF THE APPLICANT UNDER 37 C.F.R. §1.313(a)

This Petition is submitted to withdraw from issue the above-identified application at the initiative of the applicant under 37 C.F.R. §1.313(a). A Notice of Allowance was issued on April 20, 2004 by the United Patent Office in connection with the above-identified application requiring applicants to submit (i) the issue fee and (ii) new formal drawings by the July 20, 2004 deadline for doing so. A copy of the April 20, 2004 Notice of Allowance is attached hereto as **EXHIBIT A.** The deadline for paying the issue fee is July 20, 2004. Applicants have not yet paid the issue fee for the

subject application, and are filing this Petition prior to the payment of issue fee. Therefore, this Petition is timely submitted.

REMARKS

Related Facts

On March 10, 2004, the undersigned's office received a letter dated March 5, 2004 from our Japanese associates, Suzuye & Suzuye, Tokyo, Japan, addressed to Mark A. Farley, Esq., an attorney formerly, but not presently affiliated with the undersigned's law firm. A copy of an Official Action issued March 2, 2004 by the Japanese Patent Office in connection with corresponding Japanese Patent Application No. 511426/94 was enclosed with this letter. In their March 5, 2004 letter, our Japanese associates indicated that an English translation of the Official Action and copies of any cited references would be sent to us shortly thereafter. On March 17, 2004, Mr. Farley forwarded a copy of this letter to the client, and to legal representatives of a licensee and a sublicensee.

On March 29, 2004, the undersigned's office received a letter dated March 26, 2004 from our Japanese associates addressed to Mr. Farley forwarding an English translation of the March 2, 2004 Official Action, attached hereto as **EXHIBIT 5** to the Supplemental Information Disclosure Statement (**EXHIBIT C**), and copies of the four references listed below:

1. Slusher, B.S., Robinson, M.B., Tsai, G., Simmons, M.L., Richards, S.S., and Coyle, J.T., "Rat Brain *N*-Acetylated α-Linked Acidic Dipeptidase Activity", The Journal of Biological Chemistry, 265(34):21297-21301 (December 5, 1990) (hereafter referred to as "the Slusher et al. reference", attached hereto as **EXHIBIT 1** to the Supplemental Information Disclosure Statement (**EXHIBIT C**));

2. Carter, R.E., Feldman, A.R., and Coyle, J.T., "Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase", Proc. Natl. Acad. Sci. USA, 93:749-753 (January 1986) (attached hereto as **EXHIBIT 2** to the Supplemental Information Disclosure Statement (**EXHIBIT C**));

3. Halsted, C.H., Ling, E., Luthi-Carter, R., Villanueva, J.A., Gardner, J.M., and Coyle, J.T., "Folypoly-γ-glutamate Carboxylpeptidase from Pig Jejunum", The Journal Of Biological Chemistry, 273(32):20417-20424 (August 7, 1998) (attached hereto as **EXHIBIT 3** to the Supplemental Information Disclosure Statement (**EXHIBIT C**)); and

4. Wang, T.T.Y., Chandler, C.J., and Halsted, C.H., "Intracellular Pteroylpolyglutamate Hydrolase from Human Jejunal Mucosa", The Journal Of Biological Chemistry, 261(29):13551-13555 (October 15, 1986) (attached hereto as **EXHIBIT 4** to the Supplemental Information Disclosure Statement (**EXHIBIT C**)).

On March 30, 2004, Mr. Farley's affiliation with the undersigned's law firm ended. Another attorney, Cindy Yang, Esq., prepared a letter dated April 6, 2004 for signing by the undersigned which forwarded to the client, and to legal representation of the licensee and the sublicensee a copy of the March 26, 2004 letter and copies of the cited references. This letter failed to indicate that the references needed to be reviewed and possibly be made of record in the subject application.

On June 4, 2004, Edward Gates, Esq., an attorney representing a sublicensee notified the undersigned that the references cited in the March 2, 2004 Official Action issued by the Japanese Patent Office should be considered in terms of making them of record. However, since these references were first cited in a March 2, 2004 Official Action issued by the Japanese Patent Office, the deadline for submitting these references in a Supplemental Information Disclosure Statement under 37 C.F.R. §1.97(d) was June 2, 2004. As a result, the undersigned could not make the required statement under 37 C.F.R. §1.97(e), and hence, could not submit these references in a Supplemental Information Disclosure Statement to the U.S. Patent and Trademark Office.

Further to a June 10, 2004 telephone conversation between William Dixon, Jr., a Special Program Examiner in the Technology Center of Group 1600, and Ms. Yang, and a June 10, 2004 telephone conversation between Jasemine Chambers, a Director of Technology Center 1600, and Ms. Yang, applicants are filing this Petition and are concurrently filing an Amendment After Notice Of Allowance Pursuant To

37 C.F.R. §1.312, attached hereto as **EXHIBIT B**. The Amendment seeks to (1) amend the allowed claims to recite the term "monoclonal" now found in an allowed multiple dependent claim which is being canceled. A copy of this Petition has been submitted to the Examiner with the Rule 312 Amendment.

Action Requested

Applicants hereby petition that the subject application be withdrawn from issue under 37 C.F.R. §1.313(a) for consideration of the Supplemental Information Disclosure Statement under 37 C.F.R. §1.97, attached hereto as **EXHIBIT C**, including the Form PTO-1449 attached thereto as **EXHIBIT A** to the Supplemental Information Disclosure Statement, so that references not previously of record may be considered and made of record by the Patent Office.

As confirmed during a June 8, 2004 telephone conversation between Petitions Examiner Mary-Ann Morgan of the Office of Petitions at the U.S. Patent and Trademark Office and Ms. Yang, a petition under 37 C.F.R. §1.313(a) must be accompanied by:

(1) the petition fee set forth in 37 C.F.R. §1.17(h), and
(2) a showing of good and sufficient reasons why withdrawal of the application from issue is necessary.

According to 37 C.F.R. §1.17(h), the required fee for filing a petition under 37 C.F.R. §1.313(a) is ONE-

HUNDRED AND THIRTY DOLLARS ($130.00). Authorization is hereby given to charge the $130.00 fee for filing this Petition to Deposit Account No. 03-3125.

Applicants are petitioning that the subject application be withdrawn from issue in order that the four references submitted in the attached Supplemental Information Disclosure Statement may be considered by the Patent Office.

As mentioned above, applicants are concurrently filing an Amendment After Notice Of Allowance Pursuant To 37 C.F.R. §1.312 which seeks to (1) amend the allowed claims currently reciting "antibody" to incorporate the term "monoclonal" currently present in an allowed multiple dependent claims which is being canceled. In accordance with their duty of disclosure under 37 C.F.R. §1.56, applicants are filing this Petition in order to have the subject application withdrawn from issue in order that a Supplemental Information Disclosure Statement containing the four references cited in the March 2, 2004 Official Action may be considered by the Patent Office.

Applicants also point out that the filing date of the subject application is June 6, 1995, and therefore, the subject application qualifies as a Pre-GATT application. As a Pre-GATT application, any patent issuing from the subject application is entitled to a patent term of 17 years from the date of issue.

Due to the Pre-GATT status of this application, it would be prejudicial and an undue hardship to the applicants to

have to file a Request For Continued Examination (RCE) to have these four references considered by the Patent Office since filing such an RCE would cause the application to lose its Pre-GATT status, and therefore, resulting in a dramatic loss of patent term of at least nine years.

Applicants note that this Petition is being filed promptly after they became aware of the need to submit these references.

Applicants maintain that they have paid the required fee for filing this Petition and made a showing of good and sufficient reasons why withdrawal of the application from issue is necessary, and therefore, request in the interest of justice that this Petition be granted.

Summary

For the foregoing reasons, applicants earnestly solicit an expeditious withdraw from issue of the subject application under 37 C.F.R. §1.313(a) and look forward to receiving from the Director of Technology Center 1600 a communication to this effect.

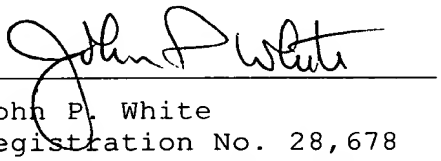
If this Petition is not granted by the July 30, 2004 deadline for paying the issue fee, applicants will pay the issue fee to avoid abandonment of this subject application, as provided in M.P.E.P. § 1308.

If a telephone conference would be of assistance in advancing prosecution of the subject application,

applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee, other than the $130.00 fee for filing a petition to withdraw from issue under 37 C.F.R. §1.313(a), is deemed necessary in connection with the filing of this petition. If any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,



John P. White
Registration No. 28,678
Attorney for Applicants
Cooper & Dunham, LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

7590 04/20/2004

JOHN P WHITE
COOPER AND DUNHAM
1185 AVENUE OF THE AMERICAS
NEW YORK, NY 10036

EXAMINER	
GUCKER, STEPHEN	
ART UNIT	PAPER NUMBER
1647	

DATE MAILED: 04/20/2004

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
08/470,735	06/06/1995	RON S. ISRAELI	41426-D/JPW/	7764

TITLE OF INVENTION: ANTIBODIES TO PROSTATE-SPECIFIC MEMBRANE ANTIGEN

APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$665	$0	$665	07/20/2004

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE REFLECTS A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE APPLIED IN THIS APPLICATION. THE PTOL-85B (OR AN EQUIVALENT) MUST BE RETURNED WITHIN THIS PERIOD EVEN IF NO FEE IS DUE OR THE APPLICATION WILL BE REGARDED AS ABANDONED.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:
A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status is changed, pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above and notify the United States Patent and Trademark Office of the change in status, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check the box below and enclose the PUBLICATION FEE and 1/2 the ISSUE FEE shown above.

☐ Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

II. PART B - FEE(S) TRANSMITTAL should be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). Even if the fee(s) have already been paid, Part B - Fee(s) Transmittal should be completed and returned. If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PTOL-85 (Rev. 11/03) Approved for use through 04/30/2004.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
08/470,735	06/06/1995	RON S. ISRAELI	41426-D/JPW/	7764

7590 04/20/2004

JOHN P WHITE
COOPER AND DUNHAM
1185 AVENUE OF THE AMERICAS
NEW YORK, NY 10036

EXAMINER	
GUCKER, STEPHEN	

ART UNIT	PAPER NUMBER
1647	

DATE MAILED: 04/20/2004

Determination of Patent Term Extension or Adjustment under 35 U.S.C. 154 (b)
(application filed prior to June 8, 1995)

This patent application was filed prior to June 8, 1995, thus no Patent Term Extension or Adjustment applies.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (703) 305-1383. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at (703) 305-8283.

PTOL-85 (Rev. 11/03) Approved for use through 04/30/2004.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (703) 746-4000

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 4 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Legibly mark-up with any corrections or use Block 1)

7590 04/20/2004

JOHN P WHITE
COOPER AND DUNHAM
1185 AVENUE OF THE AMERICAS
NEW YORK, NY 10036

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
08/470,735	06/06/1995	RON S. ISRAELI	41426-D/JPW/	7764

TITLE OF INVENTION: ANTIBODIES TO PROSTATE-SPECIFIC MEMBRANE ANTIGEN

APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$665	$0	$665	07/20/2004

EXAMINER	ART UNIT	CLASS-SUBCLASS
GUCKER, STEPHEN	1647	424-138100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list (1) the names of up to 3 registered patent attorneys or agents OR, alternatively, (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the USPTO or is being submitted under separate cover. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent); ❑ individual ❑ corporation or other private group entity ❑ government

4a. The following fee(s) are enclosed:

❑ Issue Fee

❑ Publication Fee

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s):

❑ A check in the amount of the fee(s) is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The Director is hereby authorized by charge the required fee(s), or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

Director for Patents is requested to apply the Issue Fee and Publication Fee (if any) or to re-apply any previously paid issue fee to the application identified above.

(Authorized Signature) (Date)

NOTE; The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

TRANSMIT THIS FORM WITH FEE(S)

RULE 1.116 AMENDMENT
EXPEDITED PROCEDURE
GROUP ART UNIT 1647

Dkt. 1769/41426-D/JPW/CY

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Ron S. Israeli et al.

Serial No.: 08/470,735 Examiner: S. Gucker

Filed : June 6, 1995 Group Art Unit: 1647

For : PROSTATE-SPECIFIC MEMBRANE ANTIGEN

1185 Avenue of the Americas
New York, NY 10036
June 16, 2004

Date of Notice Of Allowance: April 20, 2004 **Confirmation No.: 7764**

VIA HAND
U.S. Patent and Trademark Office
220 20th Street S.
Customer Window, Mail Stop Issue Fee
Crystal Plaza Two, Lobby, Room 1B03
Arlington, VA 22202

MAIL STOP ISSUE FEE

Sir:

AMENDMENT AFTER NOTICE OF ALLOWANCE PURSUANT TO 37 C.F.R. §1.312

This Amendment is submitted pursuant to 37 C.F.R. §1.312 in order to amend claims 128-138 and 141 to recite the term "monoclonal" previously recited in allowed multiply dependent claim 140 which would now be canceled in the above-identified application. The issue fee is due July 20, 2004 and has not yet been paid. Accordingly, this Amendment is being timely filed.

Please amend the subject application as follows:

Amendment to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of the Claims:

1-127. (Canceled)

128. (Currently Amended) A purified monoclonal antibody which binds to a fragment of an outer membrane domain of prostate specific membrane antigen, which fragment has within its structure the consecutive amino acid sequence Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID NO: 35).

129. (Currently Amended) A purified monoclonal antibody which binds to a fragment of an outer membrane domain of prostate specific membrane antigen, which fragment has within its structure the consecutive amino acid sequence Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID NO: 36).

130. (Currently Amended) A purified monoclonal antibody which binds to a fragment of an outer membrane domain of prostate specific membrane antigen, which fragment has within its structure the consecutive amino acid sequence Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID NO: 37).

131. (Currently Amended) A purified monoclonal antibody which binds to a fragment of an outer membrane domain of prostate specific membrane antigen, which fragment has within its structure each of the following amino acid sequences:

(a) Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID NO: 35);

(b) Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID NO: 36);

(c) Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID NO: 37); and

(d) Ala-Gly-Ala-Leu-Val-Leu-Ala-Gly-Gly-Phe-Phe-Leu-Leu-Gly-Phe-Leu-Phe (SEQ ID NO:38).

132. (Currently Amended) A purified monoclonal antibody which binds to a fragment of an outer membrane domain of prostate specific membrane antigen, which fragment has within its structure each of the following amino acid sequences:

(a) Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID NO: 35);

(b) Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID NO: 36); and

(c) Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID NO: 37).

133. (Currently Amended) A purified monoclonal antibody which binds to a fragment of prostate specific membrane antigen, which fragment corresponds to a hydrophilic region of an outer membrane domain of prostate specific membrane antigen, the amino acid sequence of which antigen is set forth in SEQ ID NO:2.

134. (Currently Amended) A purified monoclonal antibody which binds to a hydrophilic region of an outer

membrane domain of prostate specific membrane antigen, which hydrophilic region has within its structure the consecutive amino acid sequence Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID NO: 35).

135. (Currently Amended) A purified monoclonal antibody which binds to a hydrophilic region of an outer membrane domain of prostate specific membrane antigen, which hydrophilic region has within its structure the consecutive amino acid sequence Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID NO: 36).

136. (Currently Amended) A purified monoclonal antibody which binds to a hydrophilic region of an outer membrane domain of prostate specific membrane antigen, which hydrophilic region has within its structure the consecutive amino acid sequence Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID NO: 37).

137. (Currently Amended) A purified monoclonal antibody which binds to an outer membrane domain of prostate specific membrane antigen, the amino acid sequence of which antigen is set forth in SEQ ID NO:2.

138. (Currently Amended) A purified monoclonal antibody which binds to a hydrophilic region of an outer membrane domain of prostate specific membrane antigen, the amino acid sequence of which antigen is set forth in SEQ ID NO:2.

139. (Canceled)

140. (Canceled)

141. (Currently Amended) A composition of matter comprising the monoclonal antibody of any one of claims 128-138 and an agent conjugated to the monoclonal antibody.

142. (Previously Presented) The composition of matter of claim 141, wherein the agent is a radioisotope or toxin.

143. (Previously Presented) A composition comprising a carrier and the composition of matter of claim 141.

144. (Previously Presented) A method of imaging prostate cancer in a subject which comprises administering to the subject the composition of matter of claim 141, wherein the agent is an imaging agent under conditions permitting formation of a complex between the composition of matter and prostate specific membrane antigen, and obtaining an image of any complex so formed.

145.-160. (Canceled)

161. (Previously Presented) A monoclonal antibody having an antigen-binding region specific for the extracellular domain of prostate specific membrane antigen, the amino acid sequence of which antigen is set forth in SEQ ID NO:2.

162. (Canceled)

163. (Canceled)

REMARKS

A Notice of Allowability and a Notice of Allowance was issued on April 20, 2004 in connection with the subject application. The April 20, 2004 Notice of Allowability indicates that the allowed claims are claims 128-144 and 161 and that these claims have been renumbered as claims 1-17 for issue. However, applicants understand that this is a clerical error, and that the Notice of Allowability should indicate that claims 128-138, 140-144 and 161 have been allowed and will issue as claims 1-17.

Applicants hereinabove have amended claims 128-138 and 141 and canceled claim 140. Accordingly, upon entry of this Amendment, applicants maintain that amended claims 128-138, 141-144 and 161 will be pending and should be allowed.

Applicants maintain that the amendments to claims 128-138 and 141 do not raise any issue of new matter, and that claims 128-138 and 141, as amended, are fully supported by the specification as originally filed.

Applicants submit this Amendment to amend claims 128-138 and 141 to recite the term "monoclonal" in allowed multiply dependent claim 140 which is being canceled concurrently.

Applicants are concurrently filing a Petition To Withdraw From Issue At The Initiative Of The Applicant Under 37 C.F.R. §1.313(a) in order to have the subject application withdrawn from issue in order that a Supplemental Information Disclosure

Statement disclosing four references be considered. A copy of this Petition is attached hereto as **EXHIBIT A.**

A copy of the Supplemental Information Disclosure Statement submitted as **EXHIBIT C** with the Petition is also enclosed. In the Supplemental Information Disclosure Statement, applicants bring to the Examiner's attention the following four references which were cited in a March 2, 2004 Official Action issued by the Japanese Patent Office in corresponding Japanese Application No. 511426/94, a copy of which is attached hereto as **EXHIBIT 5** to the Supplement Information Disclosure Statement:

1. Slusher, B.S., Robinson, M.B., Tsai, G., Simmons, M.L., Richards, S.S., and Coyle, J.T., "Rat Brain *N*-Acetylated α-Linked Acidic Dipeptidase Activity", The Journal of Biological Chemistry, 265(34):21297-21301 (December 5, 1990) (attached hereto as **EXHIBIT 1** to the Supplemental Information Disclosure Statement);

2. Carter, R.E., Feldman, A.R., and Coyle, J.T., "Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase", Proc. Natl. Acad. Sci. USA, 93:749-753 (January 1986) (attached hereto as **EXHIBIT 2** to the Supplemental Information Disclosure Statement);

3. Halsted, C.H., Ling, E., Luthi-Carter, R., Villanueva, J.A., Gardner, J.M., and Coyle, J.T., "Folypoly-γ-glutamate Carboxylpeptidase from Pig Jejunum", The Journal Of Biological Chemistry, 273(32):20417-20424

(August 7, 1998) (attached hereto as **EXHIBIT 3** to the Supplemental Information Disclosure Statement); and

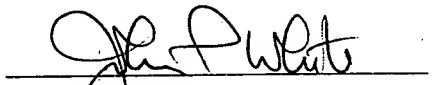
4. Wang, T.T.Y., Chandler, C.J., and Halsted, C.H., "Intracellular Pteroylpolyglutamate Hydrolase from Human Jejunal Mucosa", The Journal Of Biological Chemistry, 261(29):13551-13555 (October 15, 1986) (attached hereto as **EXHIBIT 4** to the Supplemental Information Disclosure Statement).

Applicants maintain that the amendments to the claims require no substantial amount of work on the part of the Patent Office, and merely incorporate the term "monoclonal" recited in already allowed claim 140.

If a telephone conference would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee is deemed necessary in connection with the filing of this Amendment. If any fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,



John P. White
Registration No. 28,678
Attorney for Applicants
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(212) 278-0400

Dkt. 41426-D/JPW/CY

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Ron S. Israeli et al.

Serial No.: 08/470,735 Group Art Unit: 1647

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Sir:

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

In accordance with their duty of disclosure under 37 C.F.R. §1.56, applicants would like to direct the Examiner's attention to the following disclosure which is listed on the attached Form PTO-1449 (**EXHIBIT A**). A copy of the disclosures listed below as item 1-4 are attached hereto as **EXHIBITS 1-4**:

1. Slusher, B.S., Robinson, M.B., Tsai, G., Simmons, M.L., Richards, S.S., and Coyle, J.T., "Rat Brain *N*-Acetylated α-Linked Acidic Dipeptidase Activity", The Journal of Biological Chemistry, 265(34):21297-21301 (December 5, 1990) (**EXHIBIT 1**);

2. Carter, R.E., Feldman, A.R., and Coyle, J.T., "Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase", Proc. Natl. Acad. Sci. USA, 93:749-753 (January 1986) (**EXHIBIT 2**);

3. Halsted, C.H., Ling, E., Luthi-Carter, R., Villanueva, J.A., Gardner, J.M., and Coyle, J.T., "Folypoly-γ-glutamate Carboxylpeptidase from Pig Jejunum", The Journal Of Biological Chemistry, 273(32):20417-20424 (August 7, 1998) (**EXHIBIT 3**); and

4. Wang, T.T.Y., Chandler, C.J., and Halsted, C.H., "Intracellular Pteroylpolyglutamate Hydrolase from Human Jejunal Mucosa", The Journal Of Biological Chemistry, 261(29):13551-13555 (October 15, 1986) (**EXHIBIT 4**).

An Official Report was issued on March 2, 2004 in connection with related Japanese Application No. 511426/94. A copy of the English translation of the March 2, 2004 is attached hereto as **EXHIBIT 5**. The above-listed reference was cited in that Official Action.

If a telephone conference would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee is believed necessary in connection with the filing of this Supplemental Information Disclosure Statement. However, if any fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,

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Form PTO-1449 U.S. Department of Commerce Patent and Trademark Office INFORMATION DISCLOSURE CITATION (Use several sheets if necessary)	Atty. Docket No. 41426-D/JPW/CY	Serial No. 08/470,735
	Applicant(s) Ron S. Israeli et al.	
	Filing Date June 6, 1995	Art Unit 1647

U.S. PATENT DOCUMENTS

Examiner Initials	Exh No.	Document Number	Date	Name	Class	Subclass	Filing Date If Appropriate

FOREIGN PATENT DOCUMENTS

		Document Number	Date	Country	Class	Subclass	Translation	
							Yes	No

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	1	Slusher, B.S., Robinson, M.B., Tsai, G., Simmons, M.L., Richards, S.S., and Coyle, J.T., "Rat Brain *N*-Acetylated α-Linked Acidic Dipeptidase Activity", The Journal of Biological Chemistry, 265(34):21297-21301 (December 5, 1990) (**EXHIBIT 1**);
	2	Carter, R.E., Feldman, A.R., and Coyle, J.T., "Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase", Proc. Natl. Acad. Sci. USA, 93:749-753 (January 1986) (**EXHIBIT 2**);
	3	Halsted, C.H., Ling, E., Luthi-Carter, R., Villanueva, J.A., Gardner, J.M., and Coyle, J.T., "Folypoly-γ-glutamate Carboxylpeptidase from Pig Jejunum", The Journal Of Biological Chemistry, 273(32):20417-20424 (August 7, 1998) (**EXHIBIT 3**); and
	4	Wang, T.T.Y., Chandler, C.J., and Halsted, C.H., "Intracellular Pteroylpolyglutamate Hydrolase from Human Jejunal Mucosa", The Journal Of Biological Chemistry, 261(29):13551-13555 (October 15, 1986) (**EXHIBIT 4**).

EXAMINER	DATE CONSIDERED

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP § 609: Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Ref. 1

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 265, No. 34, Issue of December 5, pp. 21297-21301, 1990
Printed in U.S.A.

Rat Brain *N*-Acetylated α-Linked Acidic Dipeptidase Activity

PURIFICATION AND IMMUNOLOGIC CHARACTERIZATION*

(Received for publication, March 19, 1990)

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N-Acetylated α-linked acidic dipeptidase (NAALA dipeptidase) is a membrane-bound metallopeptidase that cleaves glutamate from the endogenous neuropeptide *N*-acetyl-L-aspartyl-L-glutamate. In this report, we have solubilized NAALA dipeptidase activity from synaptosomal membranes with Triton X-100 and purified it to apparent homogeneity by sequential column chromatography on DEAE-Sepharose, CM-Sepharose, and lentil lectin-Sepharose. This procedure resulted in a 720-fold purification with 1.6% yield. The purified enzyme migrated as a single silver-stained band on a sodium dodecyl sulfate gel with an apparent molecular weight of 94 kDa. Using an enzymatic stain to visualize NAALA dipeptidase activity within a gel matrix, we have confirmed that the 94-kDa band is, indeed, NAALA dipeptidase. The purified enzyme was characterized and found to be pharmacologically similar to NAALA dipeptidase activity described previously in synaptosomal membrane extracts. Using the purified NAALA dipeptidase as antigen, we have raised specific and high titer polyclonal antibodies in guinea pig. Immunocytochemical studies show intense NAALA dipeptidase immunoreactivity in the cerebellar and renal cortices.

Electrophysiologic, lesion, and immunocytochemical studies suggest that the endogenous neuropeptide, *N*-acetyl-L-aspartyl-L-glutamate (NAAG),[1] may act as a neurotransmitter/neuromodulator in the central nervous system (Blakely and Coyle, 1989). Recently a quisqualate (Quis)-sensitive peptidase activity was identified in brain membranes which cleaves NAAG to *N*-acetyl-L-aspartate (NAA) and glutamate. In a manner analogous to the synaptic inactivation of acetylcholine (Cooper *et al.*, 1986), it is hypothesized that this peptidase inactivates NAAG, and that the liberated glutamate is subsequently transported into synaptosomes by the previously characterized sodium-dependent high-affinity glutamate uptake site (Blakely *et al.*, 1986; Robinson *et al.*, 1987). Alternatively, NAAG may function as a precursor to glutamate, shifting the primary role of this peptidase to regulating glutamate availability.

This peptidase has been characterized in rat synaptosomal membranes (Robinson *et al.*, 1987; Blakely *et al.*, 1988). In this crude membrane preparation, the peptidase demonstrates remarkably high apparent affinity for its putative substrate NAAG, with a $K_m = 540$ nM. The enzyme is membrane-bound, stimulated by chloride ions, and inhibited by divalent metal chelators, suggesting that it is a metallopeptidase. It is enriched in synaptic plasma membranes and is primarily localized to neural tissue and kidney. Comparison of its properties to those of other known endopeptidases, aminopeptidases, dipeptidases, and acyl amino acid-releasing enzymes suggests that it is a novel peptidase (Robinson *et al.*, 1987; Blakely *et al.*, 1988). Since it is possible that NAAG is not the sole substrate for this activity *in vivo*, this peptidase was named *N*-acetylated α-linked acidic dipeptidase (NAALA dipeptidase) for its structural specificity for *N*-acetylated α-linked acidic dipeptides. Recently, it has been demonstrated that [^{3}H]NAAG is degraded by a pharmacologically similar enzyme *in vivo* (Stauch *et al.*, 1989). These data are consistent with a role for NAALA dipeptidase in the disposition of endogenous NAAG.

In this manuscript, we describe for the first time the solubilization of NAALA dipeptidase from rat membranes, its purification to apparent homogeneity, the characterization of the purified protein, the determination of its molecular weight, the production and characterization of anti-NAALA dipeptidase antibodies, and the localization of NAALA dipeptidase immunoreactivity in brain and kidney.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Chromatographic Purification of NAALA Dipeptidase—Results of the purification of rat brain NAALA dipeptidase activity are summarized in Table I. The overall purification was 720-fold with 1.6% recovery, yielding 2 mg of highly purified NAALA dipeptidase from 500 whole rat brains. Details of the solubilization and chromatographic steps are found in the miniprint supplement.

Analysis of Enzyme Homogeneity—Fig. 2 shows SDS polyacrylamide gel electrophoresis of pooled fractions at various stages in the purification. After the lentil Lectin step, there was one major silver-stained protein band migrating at 94

* This research was supported by United States Public Health Service Grant NS 13584, a grant from the Surdna Foundation, a McKnight Scholars Award (to J. T. C.), National Institutes of Health Fellowship NS 07870 (to M. B. R.), and National Institutes of Health/National Institute of General Medical Sciences Pharmacology Training Grant 2 T32 GM07626-11 (to B. S. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: NAAG, *N*-acetyl-L-aspartyl-L-glutamate; NAALA dipeptidase, *N*-acetylated α-linked acidic dipeptidase; Quis, quisqualic acid; NAA, *N*-acetyl-L-aspartate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; ELISA, enzyme linked immunosorbent assay.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," and Fig. 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE I

Summary of the purification of NAALA dipeptidase

NAALA dipeptidase was purified from 500 whole rat brains as described under "Experimental Procedures."

Step	Protein	Specific activity	Recovery	Purification
	mg	*pmol/mg/min*	*%*	*-fold*
Crude homogenate	87,000	5	100	1
Lysed synaptosomal membranes	9,000	15	31	3
Solubilized protein	2,000	71	33	14
Pooled DEAE fractions	450	160	17	32
Pooled CM fractions	20	900	4.1	180
Pooled lentil lectin fractions	2	3,600	1.6	720

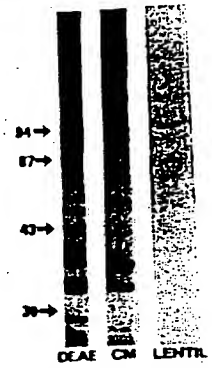


FIG. 2. SDS-polyacrylamide gel electrophoretic analysis of pooled fractions from steps during the purification of NAALA dipeptidase. *Lane A*, pooled DEAE peak I protein (10 μg of protein); *lane B*, pooled CM protein (5 μg of protein); *lane C*, pooled lentil lectin protein (0.5 μg of protein). Electrophoresis was run on a 12% gel at a constant current of 40 mA. Proteins were developed with silver staining (Wray *et al.*, 1981).

kDa, and a minor (diffuse) band migrating between 54 and 66 kDa. The literature suggests that minor contaminating protein(s) are either mercaptoethanol artifacts (Guevara *et al.*, 1982; Tasheva and Dessev, 1983) or skin keratins (Ochs, 1983).

The staining intensity of this 94-kDa band was correlated with the amount of NAALA dipeptidase activity applied to the gel. Gel electrophoresis of fractions surrounding a NAALA dipeptidase peak of activity from DEAE-Sepharose, CM-Sepharose, and size exclusion columns ($n = 4$) demonstrated that, in all cases, the 94-kDa protein band was the only band observed whose staining density coincided with NAALA dipeptidase activity (data not shown).

Although these data provide compelling evidence that the 94-kDa band is NAALA dipeptidase, it is still possible that NAALA dipeptidase is not represented by any band on the gel. Therefore, a specific enzymatic activity stain was devised to visualize NAALA dipeptidase activity within a polyacrylamide gel (Sugiura *et al.*, 1977; see "Experimental Procedures"). Since a protein separated by an SDS gel (*e.g.* denatured enzyme) is highly unlikely to exhibit enzymatic activity, partially purified NAALA dipeptidase was electrophoretically separated on a nondenaturing gel and then stained for activity. Only one band was identified having NAALA dipeptidase activity (Fig. 3*A*). Since it is not possible to accurately determine molecular weight on a nondenaturing gel, this active band was excised, homogenized, and subjected to SDS-polyacrylamide gel electrophoresis (Fig. 3*B*, *left lane*). Although

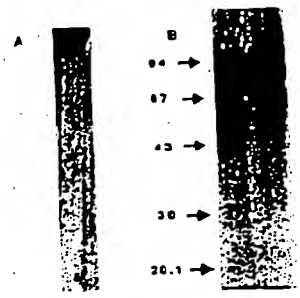


FIG. 3. Visualization of NAALA dipeptidase activity in an acrylamide gel. *A*, nondenatured gel of a semi-purified preparation stained for NAALA dipeptidase activity as described under "Experimental Methods." The resulting active band was then excised, homogenized, boiled in SDS/mercaptoethanol for 30 min, and applied to an SDS gel. *B*, the left lane is a silver-stained SDS gel of the excised NAALA dipeptidase activity band from *A*. The *right lane* is a silver-stained SDS gel of the staining components used to visualize NAALA dipeptidase activity (*e.g.* glutamate dehydrogenase, iodonitrotetrazolium violet, phenazine methosulfate). Note that the NAALA dipeptidase activity band contains only one band, migrating at 94 kDa, which cannot be attributed to the NAALA dipeptidase activity staining components.

TABLE II

Potency of compounds for inhibition/stimulation of purified NAALA dipeptidase activity

NAALA dipeptidase activity was measured as described under "Experimental Procedures" with or without inhibitors. When EGTA was tested, no exogenous cobalt was added to the assay. Peptide inhibitors were used at their previously reported IC_{50} concentrations in lysed synaptosomal membranes (Robinson *et al.*, 1987). Data are expressed as the percent of activity determined in the absence of inhibitor (control). Results are the mean of at least two experiments performed in duplicate. Except where noted, experiments were performed with L-isomers and α-linked peptides.

Compound	Concentration	Control activity
	μM	*%*
Inhibitors (μM)		
Asp-Glu	0.30	50
Quisqualic acid	0.48	51
Glu-Glu	0.75	51
Gly-Glu	6.0	67
γ-Glu-Glu	9.5	52
Glu	31	23
Glu-Glu-Glu	62	65
N-Acetyl-Asp	100	95
Sodium phosphate	100	57
EGTA	1000	4

five bands were revealed with this procedure, all bands, except for a 94-kDa protein, were attributed to the staining components used to visualize NAALA dipeptidase activity in the nondenaturing gel (*e.g.* glutamate dehydrogenase, iodonitrotetrazolium violet, phenazine methosulfate; Fig. 3*B*, *right lane*). These data suggest that the 94-kDa band is NAALA dipeptidase.

Properties of the Purified NAALA Dipeptidase—As was observed for activity characterized in lysed synaptosomal membranes (Robinson *et al.*, 1987), purified NAALA dipeptidase was potently inhibited by quisqualate with 50% inhibition at 0.48 μM (Table II). Peptidase activity was also inhibited by phosphate and EGTA; cobalt strongly stimulated activity. Purified NAALA dipeptidase showed a high apparent affinity for NAAG hydrolysis with a K_m of 140 nM (mean of two determinations within 10%).

Structure-activity relationships of purified NAALA dipeptidase were examined using peptide analogs of NAAG and

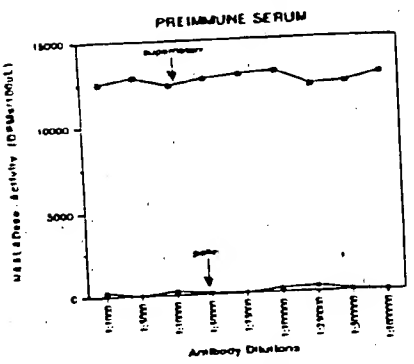
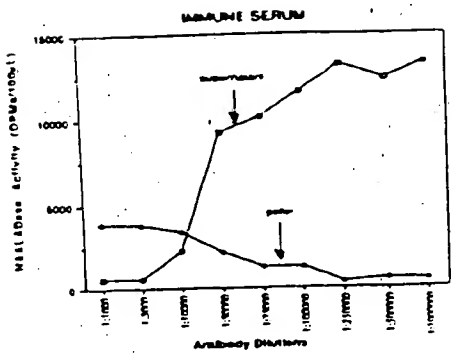


FIG. 4. Immunoprecipitation of NAALA dipeptidase activity. Details pertaining to the immunoprecipitation are described under "Experimental Methods." The graphs presented are one representative set of data obtained from three separate experiments. In brief, varying amounts of preimmune and immune sera were added to crude brain membranes and incubated overnight on a rotary shaker at 4 °C. The following day, GammaBind G-Agarose was added, incubated for 3 h at 4 °C, and centrifuged. NAALA dipeptidase activity was measured in the supernatant (□) and pellet (◆).



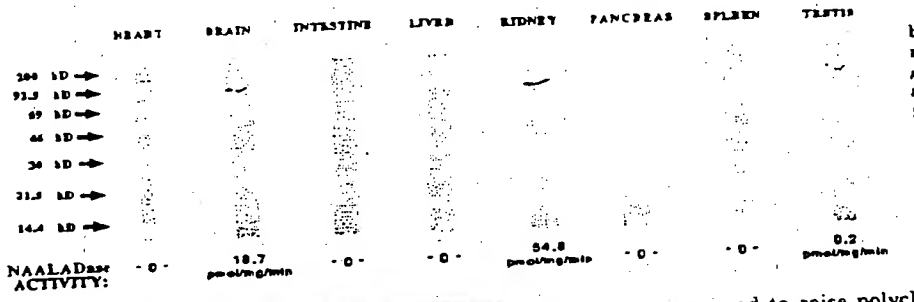


FIG. 5. Survey of the tissue distribution of NAALA dipeptidase immunoreactivity. Tissue extracts (250 μg of protein) were subject to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, incubated with immune serum against NAALA dipeptidase, followed by color development as described under "Experimental Methods." All samples shown were run in parallel. The level of NAALA dipeptidase activity identified in each region is provided.

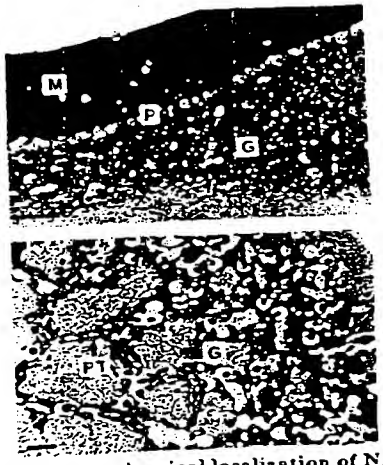


FIG. 6. Immunocytochemical localization of NAALA dipeptidase. Staining for NAALA dipeptidase immunoreactivity in rat cerebellar cortex (*top*) and rat kidney cortex (*bottom*). Note the positive staining in the molecular and granular cell layers of the cerebellar cortex and the proximal tubules and glomeruli of the kidney cortex. Details of the immunocytochemistry are described under "Experimental Procedures." Cerebellar cortex: *M*, molecular layer; *P*, Purkinje cell layer; *G*, granular layer; *Bar* = 80 μm. Kidney cortex: *PT*, proximal tubules; *G*, glomeruli; *Bar* = 300 μm.

compared with what was previously determined using a lysed synaptosomal membrane preparation (Robinson *et al.*, 1987). All peptides examined were used at their previously reported IC_{50} concentrations. The results are summarized in Table II. All data were similar to what was reported initially, except inhibition by aspartylglutamate (Asp-Glu), which was 8-fold more potent as an inhibitor of the purified enzyme than had been reported using lysed membranes (50% inhibition at 0.3 μM *versus* 2.4 μM).

Antibody Characterization—Purified NAALA dipeptidase was used to raise polyclonal antibodies in guinea pigs (see "Experimental Procedures"). Enzyme-linked immunosorbent assay, immunoprecipitation assay, and Western blotting demonstrated that the anti-NAALA dipeptidase antisera was specific and of high titer. The titer, determined by enzyme-linked immunosorbent assay (see "Experimental Procedures"), was approximately 1:10,000 (data not shown).

Immunoprecipitation—The immune serum precipitated NAALA dipeptidase activity from solubilized brain membranes; preimmune serum did not precipitate activity (Fig. 4). Immune serum partially inhibited NAALA dipeptidase activity; therefore, at antibody dilutions less than 1:100,000 total activity using the immune serum was less than activity in the preimmune control.

Western Blotting—An SDS gel loaded with crude homogenates from rat heart, brain, intestine, liver, kidney, pancreas, spleen, and testis was transferred to nitrocellulose and probed with anti-NAALA dipeptidase immune serum as described under "Experimental Procedures." The immune serum recognized a single 94-kDa band in brain, kidney, and testis only, consistent with the localization of NAALA dipeptidase activity (Fig. 5). No staining was detected using preimmune serum.

Immunocytochemistry—Immunocytochemical experiments revealed NAALA dipeptidase-positive staining in the molecular and granule cell layers of the cerebellar cortex; the Purkinje cell layer was devoid of immunoreactivity (Fig. 6, *top*). In the rat kidney, NAALA dipeptidase-positive staining was detected in the proximal tubules and glomeruli of the renal cortex; the distal tubules were essentially devoid of immunoreactivity (Fig. 6, *bottom*). No immunostaining was revealed using preimmune serum, even at 10-fold higher concentrations. In addition, preadsorption of the antiserum with purified NAALA dipeptidase (0.03 mg/ml) completely abolished immunochemical staining.

DISCUSSION

Brain NAALA dipeptidase activity was solubilized with Triton X-100 and sequentially purified with ion-exchange and

lentil lectin affinity chromatography (Fig. 1). DEAE-Sepharose resolved NAALA dipeptidase activity into two peaks; however, both peaks were pharmacologically and kinetically similar. Since DEAE peak 1 was the predominant species (>85% of eluted activity), it was employed for further purification. NAALA dipeptidase activity (peak 1) did not interact with DEAE-Sepharose at pH 7.9 and bound to CM-Sepharose at this same pH, suggesting that this protein has an unusually high isoelectric point (pI); most proteins have pI values below pH 7.4 (Righetti and Caravaggio, 1976). Chromatofocusing chromatography confirmed this finding, revealing a pI for NAALA dipeptidase of approximately pH 9.0. Following lentil lectin chromatography, the purified preparation showed one major silver-stained band on SDS-polyacrylamide gel electrophoresis migrating at 94 kDa and a minor (diffuse) band migrating between 54 and 66 kDa. The literature suggests that this minor broad band is either a mercaptoethanol artifact (Guevara *et al.*, 1982; Tasheva and Dessev, 1983) or skin keratins (Ochs, 1983). In fact, we have seen this diffuse band in lanes run with sample buffer alone.

To demonstrate that the 94-kDa protein represented NAALA dipeptidase, activity applied to an SDS gel was correlated with protein staining intensity of this band. In all gels, the 94-kDa band was the only band observed whose staining density coincided with the amount of applied NAALA dipeptidase activity. Furthermore, NAALA dipeptidase activity was visualized directly in a nondenaturing gel (Fig. 3*A*). NAALA dipeptidase activity demonstrated a low degree of migration into the nondenaturing gel, consistent with its high isoelectric point. This resulting activity band was excised from the nondenaturing gel and run on an SDS gel. Protein staining again revealed a single unique band at 94 kDa (Fig. 3*B*). Together, these data strongly suggest that this 94-kDa band is NAALA dipeptidase.

Size exclusion chromatography of the purified and semipurified protein show that NAALA dipeptidase migrates consistent with a molecular mass of 225 kDa, although larger species were occasionally observed. Both protein and activity gels demonstrate that peptidase has a denatured molecular mass of approximately 94 kDa. Therefore, it is possible the NAALA dipeptidase is a dimer composed of two identical subunits; alternatively, the larger species identified with size exclusion chromatography may represent protein-detergent complexes.

Properties of the purified protein were similar to activity previously characterized in lysed synaptosomal membranes (Robinson *et al.*, 1987), demonstrating that these properties are due to direct interaction with NAALA dipeptidase and are not indirectly mediated by other proteins present in the membrane preparation. The potent inhibition of peptidase activity by quisqualate suggests that some of its actions, which were previously attributed to interaction with a subclass of glutamate receptors (Robinson and Coyle, 1988), may be due to inhibition of NAALA dipeptidase. EGTA sensitivity and cobalt stimulation support initial data suggesting that NAALA dipeptidase was a metallopeptidase. Similar to activity in lysed synaptosomal membranes, purified NAALA dipeptidase displayed structure specificity for *N*-acetylated α-linked acidic dipeptides (Table II). Finally, the purified NAALA dipeptidase displayed a remarkably high apparent affinity for its putative substrate, NAAG, with a K_m of 140 nM.

The availability of purified protein permitted the production of anti-NAALA dipeptidase antisera. The results presented in this study demonstrate that the polyclonal antibodies raised in guinea pig are remarkably selective, of high titer, and capable of recognizing both native and denatured NAALA dipeptidase. Western analysis of gels loaded with crude brain homogenates revealed that the antisera exclusively recognized the 94-kDa band. Besides brain NAALA dipeptidase, the antibodies cross-reacted with kidney and testis NAALA dipeptidase; no immunoreactivity was observed in regions devoid of NAALA dipeptidase activity (Fig. 5). The antisera inhibited NAALA dipeptidase activity, although not completely (70% inhibition at 1:100 dilution), and was capable of precipitating NAALA dipeptidase activity from a crude brain extract (Fig. 4).

Using this selective and specific antisera, NAALA dipeptidase immunoreactivity was localized to the glomeruli and proximal tubules of the kidney cortex (Fig. 5*B*). This localization is consistent with micropunch analysis of NAALA dipeptidase activity,[3] which found that the vast majority of NAALA dipeptidase activity was localized to the renal cortex (kidney cortex = 166 pmol/mg/min *versus* kidney medulla = 10 pmol/mg/min). Interestingly, other brain peptidases, angiotensin converting enzyme and enkephalinase (Schulz, 1988; Tsuc, 1988) have also been localized in the glomeruli and proximal tubules of the kidney, areas where their putative brain substrates are not found. In the neural tissue, NAALA dipeptidase immunoreactivity was found in areas reported previously to contain NAAG (Blakely and Coyle, 1989).

NAALA dipeptidase is a novel enzymatic activity involved in NAAG hydrolysis. In this study, we have solubilized and purified rat brain NAALA dipeptidase to apparent homogeneity, developed specific anti-NAALA dipeptidase antiserum, and have begun to map its distribution in rat brain and kidney. We anticipate using the antiserum to fully determine its renal and neuronal localization and to screen a cDNA library to obtain the NAALA dipeptidase clone.

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[3] S. S. Richards, G. Forloni, M. B. Robinson, and J. T. Coyle, unpublished observation.

Supplementary Material to:

RAT BRAIN N-ACETYLATED ALPHA-LINKED ACIDIC DIPEPTIDASE ACTIVITY: PURIFICATION AND IMMUNOLOGIC CHARACTERIZATION

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EXPERIMENTAL PROCEDURES

Materials
[illegible]

NAALA dipeptidase and Protein Assays
[illegible]

Solubilization
[illegible]

Purification Scheme
[illegible]

Size Exclusion Chromatography
[illegible]

Chromatofocusing Chromatography
[illegible]

Gel Electrophoresis
[illegible]

Visualization of NAALA Dipeptidase Activity Within an Acrylamide Gel
[illegible]

Preparation of anti-NAALA Dipeptidase Antisera
[illegible]

Antibody Characterization
[illegible]

Immunohistochemistry
[illegible]

RESULTS

Effects of Cobalt on NAALA Dipeptidase Activity
[illegible]

Solubilization of NAALA Dipeptidase
[illegible]

Purification of NAALA Dipeptidase
[illegible]

Partial purification of kidney NAALA Dipeptidase activity
[illegible]

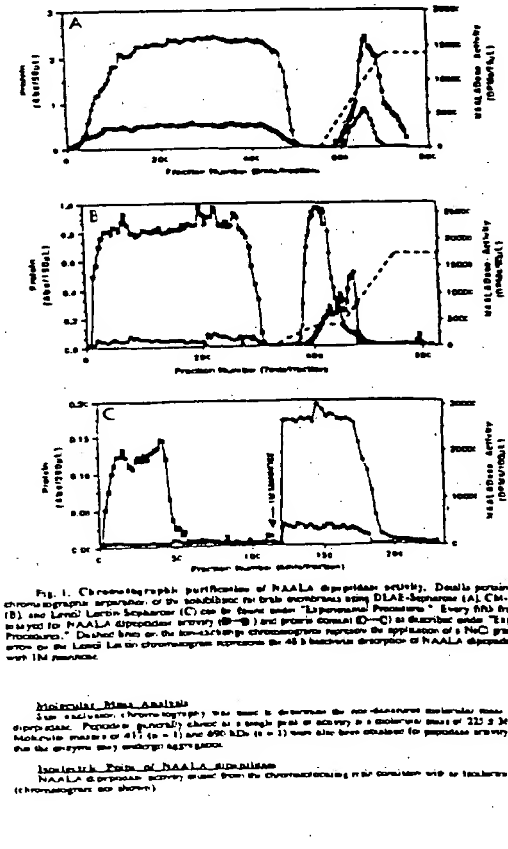

Fig. 1. Chromatographic purification of NAALA dipeptidase activity. [illegible] DEAE-Sepharose (A), CM-Sepharose (B), and Lentil Lectin Sepharose (C) [illegible]

Molecular Mass Analysis
[illegible]

Isoelectric Point of NAALA dipeptidase
[illegible]

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Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 749–753, January 1996
Neurobiology

Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase

(cancer/excitatory neurotransmission/*N*-acetylaspartylglutamate/*N*-acetylated α-linked acidic dipeptidase/glutamate)

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Communicated by Seymour S. Kety, Department of Health and Human Services, Bethesda, MD, October 20, 1995 (received for review June 18, 1995)

ABSTRACT This report demonstrates that the investigational prostatic carcinoma marker known as the prostate-specific membrane antigen (PSM) possesses hydrolytic activity with the substrate and pharmacologic properties of the *N*-acetylated α-linked acidic dipeptidase (NAALADase). NAALADase is a membrane hydrolase that has been characterized in the mammalian nervous system on the basis of its catabolism of the neuropeptide *N*-acetylaspartylglutamate (NAAG) to yield glutamate and N-acetylaspartate and that has been hypothesized to influence glutamatergic signaling processes. The immunoscreening of a rat brain cDNA expression library with anti-NAALADase antisera identified a 1428-base partial cDNA that shares 86% sequence identity with 1428 bases of the human PSM cDNA [Israeli, R. S., Powell, C. T., Fair, W. R. & Heston, W. D. W. (1993) *Cancer Res.* 53, 227–230]. A cDNA containing the entire PSM open reading frame was subsequently isolated by reverse transcription-PCR from the PSM-positive prostate carcinoma cell line LNCaP. Transient transfection of this cDNA into two NAALADase-negative cell lines conferred NAAG-hydrolyzing activity that was inhibited by the NAALADase inhibitors quisqualic acid and β-NAAG. Thus we demonstrate a PSM-encoded function and identify a NAALADase-encoding cDNA. Northern analyses identify at least six transcripts that are variably expressed in NAALADase-positive but not in NAALADase-negative rat tissues and human cell lines; therefore, PSM and/or related molecular species appear to account for NAAG hydrolysis in the nervous system. These results also raise questions about the role of PSM in both normal and pathologic prostate epithelial-cell function.

Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system and a diverse set of receptors activatable by glutamate and other glutamate-like molecules has been demonstrated to be involved in the processes of both rapid neuronal signaling and synaptic plasticity (1). Two important aspects of excitatory transmission still to be defined are the spectrum of endogenous ligands for the glutamate family of receptors and the specific mechanism(s) by which synaptic glutamate arises. It has been hypothesized that the acidic neuropeptide *N*-acetylaspartylglutamate (NAAG) might both serve as an endogenous receptor ligand and a glutamate protransmitter stored within presynaptic nerve terminals. NAAG is localized to specific groups of neurons in brain (2), from which it is released in a calcium-dependent manner upon depolarization (3, 4). One puzzling aspect of the neurophysiology of NAAG has been the reports of excitatory (5, 6), inhibitory (7, 8), and no (9, 10) electrophysiologic effects observed upon its application to different neurons. These variable effects likely reflect the diverse actions of NAAG and its metabolites at members of the glutamate receptor family.

Intact NAAG is an agonist at a subpopulation of metabotropic glutamate receptors negatively coupled to adenylate cyclase (11) and antagonizes the effects of glutamate at the *N*-methyl-D-aspartate subtype of ionotropic receptors, where it is a weak agonist (8, 12). NAAG is also catabolized to glutamate and *N*-acetylaspartate in nervous tissue by the quisqualate-sensitive *N*-acetylated α-linked acidic dipeptidase (NAALADase) (13). The hydrolysis of NAAG by NAALADase to yield glutamate may thus be an important source of this neurotransmitter. Further, NAALADase is enriched in the synaptosomal fraction of brain (14), consistent with possible roles in both the termination of NAAG-mediated actions and the regulation of glutamate concentrations at neuronal synapses.

Supporting their relevance to glutamatergic signaling, NAAG and NAALADase have been implicated in disorders of the nervous system associated with the dysregulation of glutamatergic neurotransmission, such as amyotrophic lateral sclerosis (15, 16), schizophrenia (17), and seizure disorders (18, 19). The directions of the observed alterations in NAAG levels and NAALADase activity in these disorders correlate with the predicted changes in glutamate-receptor-mediated signaling.

One form of NAALADase has been purified to apparent homogeneity from rat brain (20). The purified enzyme is a glycoprotein with a native apparent molecular mass of ~94 kDa. Specific antisera raised against the purified glycoprotein demonstrate immunoreactivity that correlates with the distribution of NAALADase activity in rat brain, peripheral nerves, kidney, and sexual organs (20–22). The following report describes our use of these antisera to identify a human cell line cDNA that encodes a hydrolase activity with the substrate and pharmacologic properties of the NAALADase previously characterized in rat brain. The discovery of a NAALADase cDNA should prove to be a substantial advance toward understanding the neurobiologic function of NAAG and its likely role in the important process of glutamatergic signaling.

MATERIALS AND METHODS

Immunoscreening. The characteristics of the anti-NAALADase antisera that were used for immunoscreening have been reported (20). Immunoscreening of a λgt11 rat brain cDNA expression library obtained from Rachael Neve (McLean Hospital, Harvard Medical School, Boston) was conducted per Young and Davis (23) with a 1:100 dilution of primary antiserum.

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Abbreviations: NAALADase, *N*-acetylated α-linked acidic dipeptidase; PSM, prostate-specific membrane antigen; NAAG, *N*-acetylaspartylglutamate; RT-PCR, reverse transcription–PCR; β-NAAG, *N*-acetyl-β-L-aspartyl-L-glutamate.

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Six immunopositive clones were subcloned into the *Eco*R1 site of pBluescript SK− (Stratagene). Plasmid DNA was isolated using the Wizard DNA purification system (Promega).

DNA Sequencing and Analysis. Dideoxynucleotide sequencing reactions were done by using Sequenase kit 70770 (Amersham) or a *Pfu* (exo-) Cyclist system (Stratagene) according to the manufacturer's instructions. Sequence analyses were conducted by using the Genetics Computer Group (Madison, WI) package, version 7.

Cell Lines. Tumor cell lines were obtained from the American Type Culture Collection. The DU145 and PC3 lines were grown in Dulbecco's minimal Eagle's medium/2 mM glutamine/10% fetal bovine serum. The LNCaP line was grown in RPMI 1640 medium/2 mM glutamine/nonessential amino acids/5% fetal bovine serum. All medium reagents were from GIBCO/BRL.

Enzyme Assays. Monolayer cultures (35-mm dish) of LNCaP, DU145, and PC3 cell lines were solubilized into 1.2 ml of ice-cold 50 mM Tris·HCl buffer (pH 7.4 at 37°C) containing 0.5% Triton X-100 by sonication. For comparative analyses, 100 μl of each cell-line lysate (20–100 μg of protein) was assayed for NAAG-hydrolyzing activity with or without the NAALADase inhibitors quisqualic acid (Research Biochemicals) (24, 25) and *N*-acetyl-β-L-aspartyl-L-glutamate (β-NAAG) (Bachem) (25). Thirty-minute radioenzymatic assays were conducted in triplicate as described by Slusher *et al.* (20) and included cell-free blanks and rat brain tissue samples as negative and positive controls, respectively. For kinetic analysis of NAALADase in the LNCaP cell line, 500 ng of total protein was assayed at substrate concentrations of 15 nM–10 μM. Reported values are the means of three assays ± SEM.

Northern Blotting. Total RNA from adult male Sprague–Dawley rats (Taconic Farms) or cell lines was prepared by the method of Chirgwin *et al.* (26). RNA was separated by electrophoresis through a 1.2% agarose gel containing 3% formaldehyde, electrophoretically transferred to a nylon membrane, and hybridized to a random-primed ^{32}P-radiolabeled cDNA probe (specific activity = 1.5×10^9 dpm/μg) prepared using a Prime-It kit (Stratagene) at 42°C overnight. Final high-stringency washes were done with 0.1× standard saline citrate/0.1% SDS at either 65°C (rat RNA) or 55°C (human cell line RNA). The RNA was determined to be intact and evenly loaded by ethidium bromide staining and/or UV shadowing. Hybridization was detected by autoradiography using a Molecular Dynamics PhosphorImager.

Reverse Transcription (RT)–PCR Cloning of Prostate-Specific Membrane Antigen (PSM) cDNA. Reverse transcription reactions were conducted at 47–50°C for 2 hr using Superscript II reverse transcriptase (BRL) according to the manufacturer's recommended conditions with the addition of recombinant RNasin (Promega) at 40 units/25 μl and in some cases the addition of 3.33 mM dimethyl sulfoxide in the RNA denaturation step (equivalent to 2 mM final concentration in the transcription reaction). PCRs were done with native *Pfu* (Stratagene) or AmpliTaq (Perkin–Elmer) polymerase according to the supplier's recommendations using a GenAmp 480 thermal cycler (Perkin–Elmer). Thermal cycling parameters consisted of an initial denaturation step (94°C for 4 min) followed by 30–35 cycles of amplification (94°C for 1 min, 60–68°C for 1 min, 72°C for 3 min), and ending in a final extension step (72°C for 7 min). Primer sequences are as follows: primer 1, TGCAGGGCTGATAAGCGAG; primer 2, AGCCACGCCACGCTCTTG; primer 3, TCATCCAATTGGATACTATG; primer 4, TCTTTCTGAGTGACATAC. Primers were designed to amplify two cDNAs containing the 5′ and 3′ regions of the PSM coding sequence [bases 134–1605 and 1143–2569 of the Israeli *et al.* (27) clone, GenBank accession no. M99487] whose overlapping sequences would contain a native *Eco*R1 restriction site (position 1572). The resultant cDNAs were joined using this *Eco*R1 site and cloned into the *Bam*HI and *Xba* I sites of the mammalian expression plasmid pcDNA3 (Stratagene), resulting in plasmid PSMA2.

Transient Transfections. The 35-mm dishes of PC3 or DU145 cells were transfected with 3 μg of supercoiled plasmid DNA [prepared by the method of Radloff *et al.* (28) using Lipofectin (BRL) per the supplier's protocol or the calcium phosphate-mediated method of Graham and van der Eb (29)]. Mock, pcDNA3CAT, and PSMA2 transfections were done in parallel, and cells were harvested 48 hr after transfection for enzymatic assays.

RESULTS

Immunoscreening with Anti-NAALADase Antisera. A screen of ≈2 million plaques of a rat brain cDNA library in λgt11 yielded six immunopositive clones of apparently similar size. Sequences of the 3′ and 5′ ends of these six cDNAs (≈250 bases) were identical. DNA sequence analysis of one of these clones (W6) revealed a 1428-bp insert that contained an open reading frame of 1407 bases. The absence of an initiation codon indicated that this cDNA did not represent a complete coding sequence. To examine size and distribution of the mRNA species represented by the W6 clone relative to NAALADase activity, we did Northern analyses of rat brain, kidney, and liver RNA using the W6 cDNA as a probe. Fig. 1 shows that the distribution of detectable transcripts corresponded to the tissue expression of NAALADase activity with brain and kidney positive and liver negative. Interestingly, at least six transcripts were detectable in brain and kidney at estimated sizes of 3900, 3000, 2800, 2100, 750, and 500 nt. This is a larger population of transcripts than would be accounted for by the NAALADase activities previously described in brain and kidney homogenates by kinetic (13) or physical analyses (20), which show one or two compartments of activity, respectively, or by immunoblots of brain that show three specific immunoreactive bands (R.E.C., unpublished data).

Comparison of the sequence of the W6 clone to the GenBank sequence data bank revealed that bases 3–1428 of this rat brain cDNA were 86% identical to bases 1106–2534 of a cDNA previously isolated from the human prostatic carcinoma cell line LNCaP (27). This identity occurs mainly in the 3′ end of the coding region of the PSM cDNA and extends 23 bases into the 3′ untranslated sequence. The function of the protein described by this cDNA was unknown; it had been identified as a potentially useful clinical marker for prostatic carcinoma and was designated PSM (27, 30).

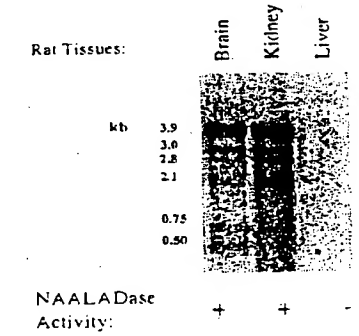

FIG. 1. Expression of putative NAALADase mRNAs in rat tissues. Electroblots of 10 μg of total RNA from rat brain, kidney, and liver were hybridized to a 1428-bp W6 cDNA probe. Note that the distribution of detectable transcripts correlates with the expression of NAALADase activity. We observe six transcripts of 3.9, 3.0, 2.8, 2.1, 0.75, and 0.5 kb in length, which are variably expressed in brain and kidney.

Coexpression of a Homologous mRNA and NAALADase Activity in Prostatic Carcinoma Cell Lines. To explore the possibility that the previously described PSM cDNA species encoded NAALADase, the LNCaP cell line from which it was cloned was assayed for NAALADase activity. Two prostatic carcinoma cell lines (DU145 and PC3) which had been reported to express neither the corresponding mRNA nor PSM antigen were assayed in parallel as negative controls. The PSM-positive LNCaP cell line exhibited high NAAG-hydrolytic activity, whereas the DU145 and PC3 (PSM-antigen-negative) lines were inactive (Table 1). Further examination of enzymatic activity in the LNCaP line revealed both high affinity and high specific activity for NAAG (K_m = 65 ± 10 nM, V_{max} = 1215 ± 40 pmol/mg of protein per min). In addition, the observed NAAG hydrolysis was inhibited by quisqualic acid, a NAALADase inhibitor. These data indicated that the LNCaP peptidase activity was NAALADase. Northern analysis of the three prostate carcinoma cell lines was subsequently conducted and, consistent with its encoding a NAALADase protein, the W6 cDNA probe detected three RNA species from the LNCaP cell line and none in the DU145 or PC3 samples (Fig. 2). The predominant RNA species expressed by LNCaP cells (2.8 kb) is the same size as the PSM antigen transcript that was detected by the PSM cDNA clone of Israeli *et al.* (27).

Expression of PSM cDNA in NAALADase-Negative Prostate Carcinoma Cell Lines Confers NAAG-Hydrolyzing Activity. With evidence that the PSM antigen cDNA might encode NAALADase, RT-PCR was used to construct a 2436-bp cDNA (PSMA2) containing the putative 2250-base PSM open reading frame [as the original reagent (27) was unavailable due to patent application]. Cells not expressing NAALADase were transiently transfected with the PSMA2 DNA and assayed for NAALADase activity. As shown in Fig. 3, the PC3 line was successfully transfected with both calcium phosphate-mediated and liposome-mediated methods and demonstrated quisqualic acid-sensitive NAAG hydrolysis. The DU145 line also demonstrated quisqualic acid-sensitive NAAG hydrolysis after transfection with the calcium phosphate-mediated method. In addition, the observed NAAG-hydrolyzing activity was sensitive to the competitive NAALADase-inhibitor β-NAAG, providing further evidence for its similarity to the activity that had originally been characterized in rat brain. Mock-transfected and control (pcDNA3-chloramphenicol acetyltransferase)-transfected cells were NAALADase-negative, ruling out induction of the enzyme by the transfection procedure.

Sequence Analysis of the PSM cDNA with Regard to NAALADase Activity. After it had been determined that the PSM cDNA encoded NAALADase, the cDNA and predicted amino acid sequences were examined for sequence similarities and structural characteristics that might be informative about the enzymatic activity of PSM. As had been reported by Israeli *et al.* (27), PSM appears to be a type II integral membrane protein, with a hydrophobic stretch of amino acids at positions

Table 1. NAALADase activity in prostatic carcinoma cell lines

Sample	[^{3}H]Glu, specific cpm	200 μM quisqualic acid
LNCaP	34,005 ± 183*	1236 ± 101†
PC3	28 ± 66	−20 ± 25
DU145	53 ± 24	−10 ± 55

Activity is expressed as specific cpm of [^{3}H]glutamate ([^{3}H]Glu) generated from [^{3}H]NAAG in 200 μl (1/10 vol) of assay reaction after subtraction of protein-free blank.

*Significantly different from protein-free blank by Student's *t* test ($P < 0.001$).

†Significantly different from LNCaP lysate alone by Student's *t* test ($P < 0.001$).

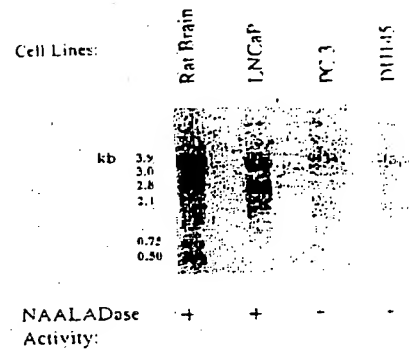

FIG. 2. Expression of putative NAALADase mRNAs in human prostatic carcinoma cell lines. Electroblots of 12 μg of total RNA from rat brain and the LNCaP, PC3, and DU145 cell lines were hybridized to a W6 cDNA probe. Among the cell lines, the distribution of detectable transcripts is limited to the NAALADase-positive LNCaP, in which 3.9-, 2.8-, and 2.1-kb species are seen.

20–43 consisting of a putative membrane-spanning domain and three arginine residues at positions 16, 17, and 19 putatively serving as a basic cytosolic anchor. The predicted molecular mass of the PSM polypeptide is 84,000 Da, similar to the size of the deglycosylated rat brain NAALADase as determined by Western analysis after pretreatment of brain homogenates with endoglycosidase F (R.E.C., unpublished data). Further, potential sites of N-linked glycosylation occur

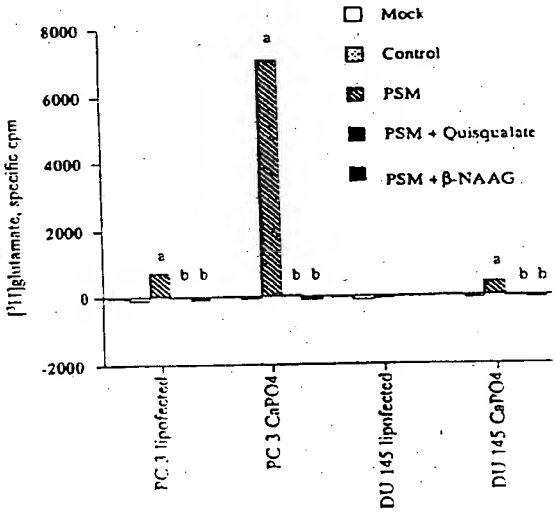

FIG. 3. NAALADase activity in PSM-transfected cell lines. One-hundred-microliter samples of PSMA2-transfected (PSM), pcDNA3-transfected (control), or mock-transfected (mock) cell lysates were assayed for NAALADase activity. Activity is expressed as described in Table 1. Error (SEM) is contained within the outlines of the bars in the graph. NAALADase inhibitors were included at a concentration of 200 μM [~50 times their IC_{50} values against rat brain NAALADase (13, 25)]. Data within each group were compared by a ANOVA with pairwise comparisons. Mock-transfected and control-transfected samples were devoid of NAAG-hydrolyzing activity relative to protein-free blanks (data not shown). a, Significantly greater than mock-transfected and control-transfected ($P < 0.001$ for all comparisons); b, significantly lower than respective PSM-transfected ($P < 0.001$ for all comparisons).

at nine positions in the putative extracellular domain of the protein. With regard to potential mechanisms for regulating NAALADase activity via posttranslational modification, a protein kinase C consensus site (31, 32) resides at Thr-14, within the short putative intracellular domain. With respect to the hydrolytic mechanism of NAALADase, the predicted amino acid PSM sequence was compared with those of other peptidase, but no functionally relevant similarities were found (33, 34).

DISCUSSION

PSM Is a NAALADase Enzyme. The search for NAAG-hydrolyzing enzymes in the rat brain led to the discovery and characterization of the quisqualic acid-sensitive, high-affinity, and high specific activity enzyme termed NAALADase (13, 24). As the transient transfection studies show, PSM exhibits important enzymatic characteristics of NAALADase as defined by the criterion of quisqualic acid-sensitive NAAG hydrolysis. Thus, our interest in NAALADase in the nervous system has led us to discover a previously uncharacterized biochemical activity of this prostate cancer marker. Interestingly, a number of hydrolases have been identified as cancer marker antigens, including another prostate cancer marker, the prostate-specific antigen, which is a serine protease (35), and another brain peptidase, enkephalinase A, which was identified independently as the common acute lymphoblastic leukemia antigen (CALLA) (36, 37).

PSM was originally identified because its expression appeared to be restricted almost exclusively to prostatic epithelial cells in human tissues as determined by immunohistochemistry with the monoclonal antibody 7E11-C5 in human tissues (30). The antigen is expressed in both normal and neoplastic prostate cells and in prostatic tumor metastases. Whereas other marker antigens for prostatic carcinoma such as prostatic acid phosphatase and the prostate-specific antigen are secreted proteins, PSM is an integral membrane protein. Thus, it is currently under investigation as a target for imaging and as a receptor for cytotoxic targeting modalities (38, 39). RT-PCR assays for PSM have also been examined for potential utility in the detection of hematogenous micrometastatic prostate cells (40).

The identification of PSM as NAALADase was originally surprising to us, given the original descriptions of the highly restricted cellular expression of PSM (30). Later reports, however, revealed that low immunoreactivity levels to the 7E11-C5 antibody and its derivative CYT-356 (37, 38) and/or mRNAs detected by RNase protection assays using PSM-derived probes (41) were found in nonprostatic human tissues, including brain. A significant discrepancy nonetheless remains between the restricted pattern of expression of PSM in human tissues as detected by the aforementioned methods and the distribution of NAALADase in rat tissues including brain, kidney, sexual organs and peripheral nerves as determined by radioenzymatic assay (13, 14, 20, 22), and immunodetection with anti-NAALADase antisera (20–22, 42). The poor correspondences between the distribution of PSM and rat NAALADase may reflect the existence of multiple NAALADase isoforms, some of which are not reactive to the available detection reagents for human PSM. Alternatively, there may be a species-related difference in NAALADase expression.

The identification of the NAALADase activity of PSM has potential impact on the study of its role in prostate biology and prostatic neoplasms. Given the localization of this enzyme to glandular epithelial cells, PSM may play a role in the local hydrolysis of peptides in prostatic fluid. Further, PSM may be responsible for the generation of extracellular glutamate in this compartment, as glutamate is known to be present in seminal fluid (43). Whether NAAG or other potential substrates for the enzyme may serve as glutamate precursors in this system remains to be determined. Additionally, the marked increase in the expression of PSM in malignant prostatic tumors raises the question of whether this cell-surface peptidase may play a role in the transformation of prostatic epithelial cells or in their ability to metastasize.

NAALADase: A Membrane Neuropeptide-Catabolizing Hydrolase. The identification of PSM as a NAALADase enzyme makes its corresponding cDNA the only NAALADase clone to have been characterized. The unique pharmacology and tissue distribution of NAALADase indicate that it is a novel member of the membrane hydrolase family (13). The hydrolysis of NAAG by NAALADase in brain membranes is inhibited at least 90% by the general metalloprotease inhibitor *o*-phenanthroline and divalent metal chelators EGTA and EDTA. Conversely, its activity is increased by supplementation of divalent metal cations (13, 20, 44). Further, NAALADase is not inhibited by general serine or activated carboxyl protease inhibitors or by thiol-modifying reagents (13). These findings led us to the hypothesis that NAALADase was a metallopeptidase. Given that we are unable to identify significant sequence similarities between NAALADase and metallopeptidases that have been previously cloned and sequenced (34), however, a definitive mechanistic categorization of the enzyme will require further study.

Analysis of the translated PSM cDNA sequence yields information about the structural elements of one form of the NAALADase enzyme. The predicted general structure composed of a short intracellular domain, a single transmembrane element, and a large globular extracellular domain is common among membrane hydrolases (45). Multiple potential N-linked glycosylation sites account for the native antigen's high carbohydrate content. Also, the protein kinase C consensus site at Thr-14 identifies one possible mechanism for the regulation of NAALADase activity. Interestingly, the arachidonic acid metabolite 12(*S*)-hydroxyeicosatetraenoic acid has been found to enhance the motility and invasiveness of rat prostate AT2.1 tumor cells via activation of protein kinase C α (46). When considered together with our results, this raises the possibility that the regulation of the enzymatic activity of PSM by protein kinase C might increase the metastatic potential of prostatic tumors. As reported by Israeli *et al.* (27), the region of the PSM cDNA from nt 1511–1961 (as denoted in the GenBank sequence) is 54% identical at the nucleic acid level to the human transferrin receptor cDNA. It is unlikely that transferrin is a required component of the active NAALADase holoenzyme, however, because NAALADase activity appears to be represented by a single protein band in both nondenaturing and denaturing polyacrylamide electrophoresis gels (20).

Synaptic hydrolases play a critical role in modifying the action of signaling molecules in the nervous system. For example, ATP released from synaptic vesicles may either activate members of the P class of purinergic receptors or may be hydrolyzed locally to adenosine, which activates purinergic receptors of the A type (47). By analogy, we envision a similar role for NAALADase in the disposition of NAAG in which the intact dipeptide could bind to NAAG-sensitive receptors or, through the action of NAALADase, NAAG could serve as a source of synaptic glutamate. Whether intact NAAG or glutamate derived from NAAG would be the active species at a given synapse would depend on the type(s) of receptors present and the expression of NAALADase activity therein. In such a dual model, the regulation of NAALADase activity could be the fulcrum determining whether NAAG- or glutamate-mediated activity would predominate after NAAG release. In addition to serving this proposed function at neuronal synapses in the central nervous system, regulation of NAAG- and/or glutamate-mediated signaling by NAALADase may extend to other tissues where it is known to be expressed, such as in the neuromuscular junction (42) or the prostate gland (30).

Implications for Future Studies of Nervous System and Prostate Biology. Identification of an enzymatic activity for PSM will facilitate the characterization of its role(s) in normal and malignant prostate cell function and further address its utility as a prostate cancer marker and targeting functionality. The discovery of a NAALADase-encoding cDNA has resulted in the identification and beginning of the molecular characterization of what appears to be a family of NAALADase-like species. Additionally, the cDNA provides a genetic tool for studying NAALADase expression at the transcriptional level, which will be particularly useful in determining its cellular distribution in the complex networks of closely apposed cells in nervous tissue.

In the broader context of excitatory neurotransmission, the characterization of NAALADase is a crucial step in understanding the disposition of NAAG and the role this neuropeptide may play in glutamatergic signaling. Moreover, the observation that dysregulation of glutamate, NAAG, and NAALADase/PSM occurs in a variety of human disorders is substantial evidence that further study of NAALADase may have important clinical applications both within and beyond the nervous system.

We thank Dr. Rachael Neve for providing the cDNA library used to isolate the W6 clone and are grateful to Drs. Neve, Christine Konradi, Steven Hyman, Urs Berger, Joseph Pearlberg, Mathew Enna, and Lila Hamill for technical advice and assistance. This research was supported by National Institutes of Health Grant MH-572901 and a National Alliance for Research on Schizophrenia and Depression Senior Investigator Award to J.T.C.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 32, Issue of August 7, pp. 20417–20424, 1998
Printed in U.S.A.

Folylpoly-γ-glutamate Carboxypeptidase from Pig Jejunum

MOLECULAR CHARACTERIZATION AND RELATION TO GLUTAMATE CARBOXYPEPTIDASE II*

(Received for publication, March 9, 1998, and in revised form, May 12, 1998)

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Jejunal folylpoly-γ-glutamate carboxypeptidase hydrolyzes dietary folates prior to their intestinal absorption. The complete folylpoly-γ-glutamate carboxypeptidase cDNA was isolated from a pig jejunal cDNA library using an amplified homologous probe incorporating primer sequences from prostate-specific membrane antigen, a protein capable of folate hydrolysis. The cDNA encodes a 751-amino acid polypeptide homologous to prostate-specific membrane antigen and rat brain *N*-acetylated α-linked acidic dipeptidase. PC3 transfectant membranes exhibited activities of folylpoly-γ-carboxypeptidase and *N*-acetylated α-linked acidic dipeptidase, while immunoblots using monoclonal antibody to native folylpoly-γ-glutamate carboxypeptidase identified a glycoprotein at 120 kDa and a polypeptide at 84 kDa. The kinetics of native folylpoly-γ-carboxypeptidase were expressed in membranes of PC3 cells transfected with either pig folylpoly-γ-carboxypeptidase or human prostate-specific membrane antigen. Folylpoly-γ-carboxypeptidase transcripts were identified at 2.8 kilobase pairs in human and pig jejunum, human and rat brain, and human prostate cancer LNCaP cells. Thus, pig folylpoly-γ-carboxypeptidase, rat N-acetylated α-linked acidic dipeptidase, and human prostate-specific membrane antigen appear to represent varied expressions of the same gene in different species and tissues. The discovery of the jejunal folylpoly-γ-carboxypeptidase gene provides a framework for future studies on relationships among these proteins and on the molecular regulation of intestinal folate absorption.

Dietary folates, a heterogeneous mixture of folylpoly-γ-glutamates, are absorbed by a two-stage process of progressive hydrolysis at the jejunal brush border membrane followed by transport of monoglutamyl folate derivatives across the intestinal mucosa (1). Previously, our laboratory (2) purified folylpoly-γ-glutamate carboxypeptidase (FGCP)[1] from human jejunal brush-border membranes as a zinc-activated exopeptidase that releases terminal glutamates sequentially and is stable at pH greater than 6.5. We identified a separate intracellular lysosomal carboxypeptidase in human jejunal mucosa that cleaves folylpoly-γ-glutamates with an endopeptidase mode of action at a pH optimum of 4.5 and that is distinguished from membranous FGCP by its complete inhibition by *p*-hydroxymercuribenzoate (3). Subsequent experiments detected the two separate folate hydrolases in intracellular and brush-border membrane fractions of pig jejunal mucosa, each with properties identical to those found in human jejunum (4). A monoclonal antibody Mab-3 to the purified pig jejunal brush-border FGCP detected a 120-kDa subunit protein that was localized by immunoreactivity to the jejunal brush-border site of *in vivo* hydrolysis of folylpoly-γ-glutamates (5).

Attempts at molecular characterization of pig jejunal FGCP were facilitated by the recent and serendipitous descriptions of the molecular properties of two other proteins, human prostate-specific membrane antigen (PSM) and rat brain *N*-acetylated α-linked acidic dipeptidase (NAALADase). The cDNAs encoding these two proteins demonstrate 87% nucleotide and 85% amino acid sequence identity (6–8) and appear to be homologues of the same enzyme. Previously, we (8, 9) showed that PC3 cells transfected with either of these cDNAs exhibit *N*-acetylaspartylglutamate (NAAG)-hydrolyzing activity characteristic of NAALADase. Others found that PC3 cells transfected with the human PSM cDNA are capable of hydrolysis of folylpoly-γ-glutamate (10) with an exopeptidase activity mechanism similar to that previously described for human jejunal FGCP (2). The discovery that the hydrolysis of both NAAG and folylpoly-γ-glutamate can be attributed to the same molecule (PSM) led to the recommendation that human PSM and rat brain NAALADase be identified under a single IUBMB-approved name (11), subsequently designated glutamate carboxypeptidase II (GCP II; EC 3.4.17.21).

The goals of the present study were to characterize the molecular structure of pig jejunal FGCP while exploring its potential genetic and biological similarities to human PSM and rat NAALADase. We found extensive molecular homology and overlapping catalytic capabilities among pig FGCP, human PSM, and rat NAALADase, consistent with the concept that the three proteins represent varied expressions of the same gene in different species and tissues. The original discovery of the pig FGCP gene provides a molecular framework for future studies on the biological relationships among these proteins and on the integration of jejunal folate hydrolysis within the overall process of the intestinal absorption of dietary folates.

* This work was supported by National Institutes of Health Grants DK-35747, DK-45301, and MH-572901. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF050502.

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[1] The abbreviations used are: FGCP, folylpoly-γ-glutamate carboxypeptidase; NAALADase, *N*-acetylated α-linked acidic dipeptidase; PSM, prostate-specific membrane antigen; NAAG, *N*-acetylated aspartylglutamate; GCP II, glutamate carboxypeptidase II; 1100, ileal 100-kDa protein; DPP IV, dipeptidyl peptidase IV; GH, glutamate hydrolase; RFC, reduced folate carrier protein; FBP, folate-binding protein; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; bp, base pair(s); kb, kilobase pair(s).

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Filed: June 6, 1995
Exhibit 3

EXPERIMENTAL PROCEDURES

Reagents—The SuperScript preamplification system was purchased from Life Technologies, Inc. *Taq* DNA polymerase was purchased from Sigma. [α-^{32}P]dCTP (3000 mCi/mmol) and [α-^{35}S]dATP (1000 mCi/mmol) were purchased from Amersham Pharmacia Biotech. A cDNA probe for human actin was obtained from CLONTECH (Palo Alto, CA). *N*-Acetylaspartyl-[3,4-^{3}H]glutamate (41.8 Ci/mmol) and α-[^{32}P]dATP (6000 Ci/mmol) were obtained from NEN Life Science Products. AG 1-X8 anion exchange resin (200–400-mesh, formate form) was purchased from Bio-Rad. 2-(Phosphonomethyl)pentanedioic acid was a gift of Dr. Barbara Slusher, Guilford Pharmaceuticals (Baltimore, MD). Folyl-γ-Glu-γ-[^{14}C]Glu was available as a prior gift from Dr. C. Krumdieck (University of Alabama Birmingham). Purified native pig jejunal FGCP and its monoclonal antibody Mab-3 were available at −70 °C from our previous experiment (5). Peptide-*N*-glycosidase F was purchased from Oxford Glyco Sciences (Bedford, MA). All other reagents were obtained from Sigma, Fisher, and various other commercial sources.

Animal and Human Tissues—Fresh jejunal and ileal mucosal scrapings were obtained from market pigs within 5 min of killing at the University of California (Davis, CA) slaughterhouse and were immediately washed in ice-cold saline, frozen in liquid nitrogen, and stored at −70 °C. They were then used for the preparation of brush-border membranes that were purified >20-fold according to appropriate marker enzymes and our previously described procedure (5). For subsequent RNA and poly(A$^+$) RNA preparations, portions of pig liver; renal cortex; and duodenal, jejunal, and ileal mucosa were frozen in liquid nitrogen and stored at −70 °C. Human jejunal segments of ~2-cm length were obtained fresh in the operating room from obese patients undergoing elective gastric bypass surgery with gastrojejunal anastomosis, according to acceptable use exemption from the University of California Davis Human Subjects Committee. Segments were opened longitudinally and were washed immediately in ice-cold 4 M guanidium thiocyanate prior to freezing in liquid nitrogen and storage at −70 °C.

Cell Lines—Tumor cell lines were obtained from the American Type Culture Collection (Rockville, MD). PC3 cells were grown in MEM supplemented with 2 mM glutamine, 10% fetal bovine serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin; LNCaP cells were cultured in RPMI supplemented with nonessential amino acids, 5% fetal bovine serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin. All media reagents were obtained from Life Technologies.

Peptide Microsequencing—As described previously, FGCP was purified from pig jejunal brush-border membranes, and the major subunit protein was identified at 120 kDa by denaturing 6% polyacrylamide gel electrophoresis and immunoblot with Mab-3 monoclonal antibody (5). A parallel gel was stained with Coomassie Blue, and the single 120-kDa band was electroeluted using the Amicon Centrilutor system (12). A peptide digest was prepared by overnight incubation of the eluate with a 50-fold molar excess of cyanogen bromide in 70% formic acid. The resultant peptide fragments were separated on a 7.5% Tricine gel and blotted to ProBlott membranes (Applied Biosystems, Foster City, CA). Peptide sequencing followed the Edman reaction, and amino acids were identified by high performance liquid chromatography (12).

Two peptide sequences contained the sequences KILIARYGKI and LTKELQ, which were 80 and 83% identical to the sequences KIVIARYGKV and LTKELK in the amino acid sequence of human PSM, respectively (6). The corresponding PSM nucleotide sequences encoding these peptides (594–624 and 1426–1446 bp (6)) were used to design sense and antisense oligonucleotide primers for the polymerase chain reaction. Approximately 10 μg of total RNA was extracted from pig jejunal mucosa using TRIzol reagent (Life Technologies) (13), and first-strand cDNA was synthesized using the SuperScript preamplification system (Life Technologies) (14). Following a polymerase chain reaction with the described primers, the amplified product was subcloned into pBluescript II (Stratagene Cloning Systems, La Jolla, CA). A subsequent dideoxy chain termination reaction (15) identified a cDNA sequence of 853 bp that had 87% nucleotide identity to the corresponding region of PSM (6).

Pig Jejunal cDNA Library Construction and Screening—Approximately 10 μg of poly(A$^+$) RNA was prepared from pig jejunal mucosal RNA by the FastTrak 2.0 poly(A$^+$) RNA isolation system (Invitrogen, Carlsbad, CA) (16) and was used for custom construction of a pig jejunal mucosal cDNA library in λZAP by Stratagene Cloning Systems, with a yield of 1.1×10^{10} plaque-forming units/ml. The cDNA library was probed with the amplified 853-bp cDNA fragment using established screening methods (17), and positive plaques were purified by secondary and tertiary screening. Following *in vivo* excision and agarose gel electrophoresis, six purified cDNA clones of different sizes between 1.6 and 2.5 kb were identified by Southern analysis using the 853-bp cDNA probe.

cDNA Sequence Analysis—Both strands from each clone were sequenced completely by the dideoxy chain termination reaction using the T3 or T7 polymerase vector primer sequences (15) and by primer walking using gene-specific oligonucleotide primers that were constructed from bases −8 to −5, 203–223, 590–605, 822–836, 948–962, 1237–1251, 1526–1540, 1647–1661, and 2078–2092 (sense) and from bases 284–303, 544–558, 786–800, 1110–1115, 1456–1470, 1645–1660, 1986–2001, and 2237–2245 (antisense). The full cDNA sequence was confirmed independently by cycle sequencing of each clone using the LI-COR 4200 automated sequencer (LI-COR, Lincoln, NE). Clone 7 incorporated all sequences represented in the others, except for an additional 46 bp in the 5′-untranslated region of clone 10 and 25 bp in the 3′-untranslated region of clone 4. No additional sequences were detected in the 5′-untranslated region by rapid amplification of cDNA ends (18). Nucleotide and amino acid sequence identities among pig FGCP, human PSM (6), rat NAALADase (7, 8), and other relevant proteins were analyzed by the BESTFIT and PILEUP programs of version 9.1 of the Genetics Computer Group sequence analysis software package (Madison, WI).

Preparation and Expression of the Cloned Enzyme—A construct of the cDNA of FGCP was prepared by *Hin*dIII and *Xba*I excision from the vector, followed by ligation into the mammalian expression vector pcDNA3 (Invitrogen). One hundred-mm dishes of PC3 cells were transfected with 25 μg of supercoiled plasmid DNA containing the cDNA of pig FGCP or human PSM (construct PSMA2) (9) using the calcium phosphate-mediated method in 50 mM Hepes buffer, pH 7.05 (19). Mock transfected PC3 cells served as controls. Cells were harvested 72 h post-transfection for enzymatic assays by scraping them into 50 mM Tris-HCl buffer (pH 7.4 at 37 °C). Membranes were prepared from the transfected and control PC3 cells by brief sonication followed by centrifugation (35,000 × *g*) for 30 min. The membrane pellets were then solubilized by sonication into 50 mM Tris-HCl plus 0.5% Triton X-100. The protein concentration of the solubilized membrane was determined using the enhanced protocol BCA assay (Pierce) or Bio-Rad kit.

Enzyme Activities—The hydrolysis of NAAG was measured in purified pig jejunal and ileal brush-border membranes and in transfected and mock transfected PC3 cell membranes by radioenzymatic assay, whereby hydrolysis was quantitated via scintillation spectrometry of [^{3}H]glutamate produced from radiolabeled substrate after separation of substrate and product by ion exchange chromatography (20). Assays were initiated by the addition of labeled NAAG at a concentration of 2.5 nM.

Folate hydrolysis was measured in membranes from PSM and FGCP transfectants and mock transfected PC3 cells using substrate folyl-γ-Glu-γ-[^{14}C]Glu and a modification (5) of the method of Krumdieck and Baugh (21) in which terminal [^{14}C]Glu is counted in a liquid scintillation counter after charcoal precipitation of unreacted substrate. Duplicate reactions used 12 μM substrate in 33 mM 3,3-dimethylglutarate buffer containing 0.1 mM zinc acetate. Initial studies evaluated pH dependence and the inhibitory effect of 0.5 mM *p*-hydroxymercuribenzoate in membranes from each cell preparation. Subsequently, kinetic properties were compared in membranes from purified pig jejunal brush borders and from FGCP and PSM transfectants by measurements over a range of substrate concentrations at pH 6.5.

Immunoblots—Membranes from the PC3 cells that were transfected with the cDNA of either human PSM or pig FGCP or that were mock transfected were solubilized in 0.1% Triton X-100. Membrane proteins from the FGCP transfectant were deglycosylated under denaturing conditions using peptide-*N*-glycosidase F according to the manufacturer's protocol. Solubilized membrane proteins and a sample of purified native pig jejunal brush-border FGCP (5) were electrophoresed in parallel on 8% SDS-polyacrylamide gels (22), followed by transfer to polyvinylidene difluoride membranes (Millipore Corp., Marlborough, MA). Protein bands were identified using the monoclonal antibody Mab-3 to the purified native pig FGCP (5) followed by a secondary goat anti-mouse antibody conjugated with alkaline phosphatase (Bio-Rad). The authenticity of Mab-3 immunoreactivity was proven previously by its ability to immunoprecipitate the 120-kDa subunit of FGCP from solubilized pig jejunal brush-border membranes and to localize FGCP in pig intestine immunohistochemically (5).

Northern Blots—Total RNA was extracted from rat brain, LNCaP cells, and pig and human jejunal mucosa (13). Poly(A$^+$) RNA was prepared from pig liver and kidney and duodenal, jejunal, and ileal mucosa (16). Human brain poly(A$^+$) RNA was obtained from CLONTECH Inc. (Palo Alto, CA). A 2.4-kb *Eag*I–*Nae*I fragment of FGCP was

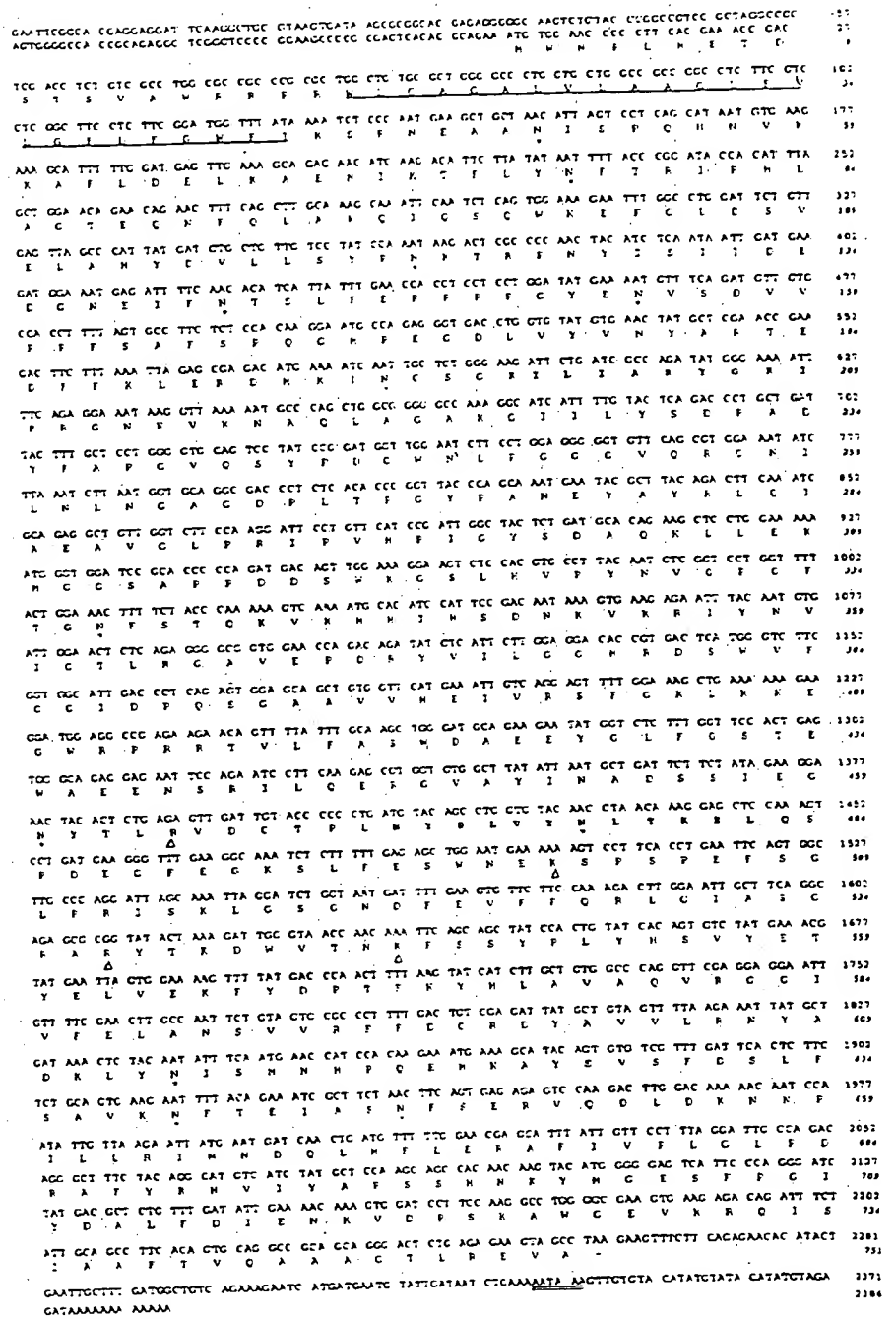

FIG. 1. **Nucleotide and amino acid sequences of pig FGCP.** Amino acid sequences that correspond with 100 and 83% identity to two peptide sequences from native pig jejunal brush-border FGCP are shown in *boldface type*. There are 146 bp in the 5'-untranslated region, 2253 bp translating to 751 amino acids in the open reading frame, and 133 bp in the 3'-end. The putative transmembrane domain (11) is *underlined*, and the 3' terminal polyadenylation signal is *double underlined*. Flanking residues Arg^{16} and Arg^{17} are conserved at the N-terminal side of the leucine-rich hydrophobic transmembrane domain between residues 20 and 43. The putative catalytic domain (11) is composed of residues 275–588. There are 12 glycosylation sites (*stars*), of which 10 are conserved in the human PSM sequence (6) and 9 are conserved in the rat NAALADase sequence (7, 8). Zinc-binding residues are conserved at His^{377}, Asp^{386}, Glu^{425}, Asp^{454}, and His^{554} (*closed circles*). Four positively charged residues predicted to be involved in substrate binding are conserved at Arg^{464}, Lys^{601}, Arg^{537}, and Lys^{546} (*open triangles*).

TABLE I

Regional peptide homologies between pig FGCP and selected proteins

The BESTFIT program was used to assess the best regional amino acid sequence similarities and identities among pig FGCP, selected other type II proteins, and other proteins relevant to folate metabolism and membrane transport.

Protein	Reference	GenBank™ accession No.	FGCP region	Similarity	Identity
				%	%
Human PSM	6	M99487	1–751	92	91
Rat NAALADase	7	U75973	1–751	88	83
Rat NAALADase	8	AF040256	1–751	88	83
Human transferrin receptor	26	M11507	9–747	44	31
V. proteolyticus	27	S24314	160–647	43	33
S. griseus	28	S66427	357–555	45	36
Rat I100	29	AF009921	20–750	50	41
Human DPP IV	30	M80536	259–711	41	29
Human GH	31	U55206	521–706	42	28
Mouse RFC	32	L23755	507–708	35	27
Pig FBP	33	U89949	4–182	38	29

purified and ^{32}P-labeled for subsequent probing of Northern blots. Pig tissue samples were also probed with a ^{32}P-labeled fragment of human actin cDNA as a positive internal control. After electrophoretic separation in 1.2% agarose, 2.2 M formaldehyde gels and transfer to nylon membranes (Schleicher & Schuell), RNA species were identified by hybridization to cDNA probes as detected autoradiographically (23).

RESULTS

Molecular Sequence of Pig Jejunal FGCP—The complete nucleotide and deduced amino acid sequences of the cDNA of pig FGCP are shown in Fig. 1. The deduced amino acid sequences KILIARYGKIF and MYSLVYNLTKELQ correspond with 100 and 85% identities to two amino acid sequences, KILIARYGKIF and MYILVYGLTKELQ, that were identified in the peptide digest of the native purified enzyme. The complete cDNA of FGCP is composed of 2532 bases: 146 in the 5′-untranslated region, 2253 in the open reading frame that encode 751 amino acids, and 133 in the 3′-untranslated region. The nucleotide and deduced amino acid sequences of pig FGCP were compared with those of human PSM (6) and rat NAALADase (7, 8). Within the open reading frame, the nucleotide identities between pig FGCP and human PSM and rat brain NAALADase were 88 and 83% respectively, while there was very little similarity in the 5′-untranslated region. The amino acid sequence of pig FGCP was 92% similar and 91% identical to that of human PSM and was 87% similar and 83% identical to that of rat NAALADase (Table I). Structural comparisons followed the recent Rawlings and Barrett analysis of human PSM and rat NAALADase (11). The Kyte and Doolittle hydropathy plot (24) of pig jejunal FGCP was identical to those of human PSM and rat NAALADase and typifies a type II protein that conserves a short N-terminal cytoplasmic region and a single hydrophobic transmembrane between residues Trp^{20} and Ile^{43}. Like human PSM and rat NAALADase, pig FGCP lacks an N-terminal signal sequence but contains positively charged residues at the N-terminal side of the transmembrane domain that are characteristic of type II membrane proteins (25), while the remainder of the molecule containing the catalytic domain occupies an extracellular site. The putative catalytic domain of human PSM and rat NAALADase is conserved in FGCP between residues 275 and 588. Twelve N*X*(S/T) potential glycosylation sites occur at Asn positions 51, 77, 122, 141, 154, 196, 337, 460, 477, 614, 639, and 646, of which 10 are conserved by human PSM and nine by rat NAALADase. Five putative catalytic zinc binding residues are conserved at positions His^{378}, Asp^{388}, Glu^{426}, Asp^{454}, and His^{554}. Within the proposed specificity pocket, four positively charged residues are conserved at Arg^{464}, Lys^{501}, Arg^{537}, and Lys^{546}.

Homologies with Other Relevant Proteins—The BESTFIT computer program was used to analyze regional amino acid sequence homologies between pig FGCP and selected structurally and functionally related proteins (Table I). In addition to extensive sequence similarities and identities among FGCP, PSM, and NAALADase, FGCP exhibited similarities with three other M28 family members: human transferrin receptor (26) and aminopeptidases from *Vibrio proteolyticus* (27) and *Streptomyces griseus* (28). Rat I100, a recently characterized ileal peptidase with type II structure (29), also shares extensive amino acid similarity with FGCP, whereas there was less sequence similarity between FGCP and human dipeptidyl peptidase IV, an enzyme that appears to be functionally related to I100 (30). The PILEUP program was used to clarify amino acid alignments within the putative catalytic regions of FGCP, rat ileal I100 (29), and human dipeptidyl peptidase IV (30). All five putative catalytic zinc binding residues (11) were conserved between pig jejunal FGCP and rat ileal I100 at His^{378}, Asp^{388}, Glu^{426}, Asp^{454}, and His^{554}, while only one zinc binding residue at Glu^{426} was conserved in dipeptidyl peptidase IV. Among the putative substrate binding basic amino acids (11) that were conserved in FGCP, PSM, and NAALADase, only Arg^{464} was conserved in I100, and only Arg^{537} was conserved in dipeptidyl peptidase IV. Several amino acids typical of a serine carboxypeptidase mechanism (29) were conserved further downstream, including Ser^{632} in all three proteins and Asp^{667} and His^{690} in FGCP and I100. Structural similarities between FGCP and selected other proteins relevant to folate hydrolysis and transport were also investigated. Human glutamate hydrolase (an intracellular peptidase capable of folylpoly-γ-glutamate hydrolysis (31)) and two proteins involved in the transport of monoglutamyl folates (the mouse reduced folate carrier protein (RFC) (32) and pig folate-binding protein (FBP) (33)) showed only weak similarities to short regions at the N- or C-terminal ends outside of the catalytic region of FGCP.

Enzyme Activities—As depicted in Fig. 2, NAALADase-specific activity was 16-fold greater in pig jejunal brush-border membranes than in ileal brush-border membranes. NAALADase was abundant in membranes from PC3 cells transfected with the cDNA of pig jejunal FGCP but was absent from control PC3 cells. Previously characterized inhibitors (9, 20) nearly eliminated NAALADase activity in jejunal brush-border membranes and in FGCP transfectant membranes but had minimal effect on NAALADase activity in ileal brush-border membranes.

As depicted in Fig. 3 (*left panel*), FGCP activity in PC3 transfectant membranes was maximal at pH 6.5 and was not inhibited by the addition of *p*-hydroxymercuribenzoate to the reaction mixture. FGCP activity with an identical pH profile and lack of *p*-hydroxymercuribenzoate inhibition was found in PC3 cells transfected with the cDNA of PSM (not shown). By contrast, folate hydrolysis was much less in membranes of

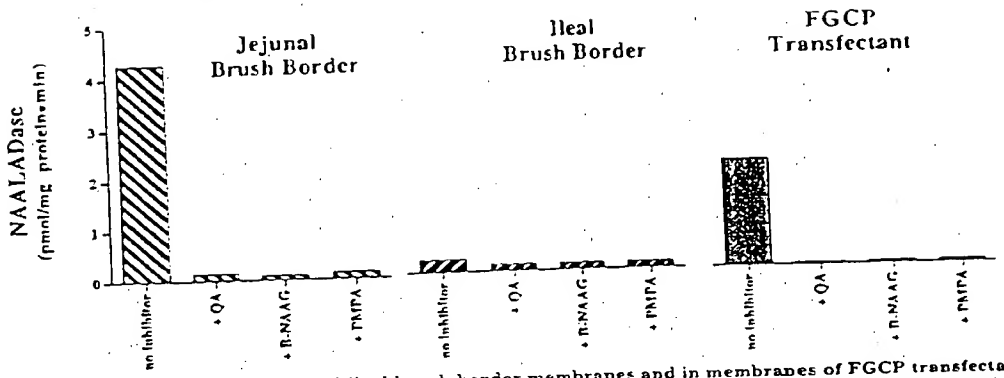

FIG. 2. NAALADase activity in pig jejunal and ileal brush-border membranes and in membranes of FGCP transfectants. Reaction mixtures included substrate NAAG (2.5 nM), jejunal brush-border membrane protein (2 μg), ileal brush-border membrane protein (20 μg), and FGCP transfectant membrane protein (2 μg), and NAAG inhibitors quisqualic acid (*QA*, 50 μM), β-*N*-acetylaspartylglutamate (*β-NAAG*, 25 μM), and 2-(phosphonomethyl)pentanedioic acid (*PMPA*, 10 nM). Data are expressed as the mean of three assays. Jejunal brush-border membranes demonstrated 16-fold greater NAAG-hydrolyzing activity than ileal brush-border membranes (4.275 ± 0.068 *versus* 0.253 ± 0.002 pmol/mg of protein·min). FGCP transfectants demonstrated NAAG-hydrolyzing activity (2.112 ± 0.077 pmol/mg of protein·min), while activity was negligible in controls (0.006 ± 0.010 pmol/mg protein·min). NAALADase inhibitors reduced NAAG hydrolysis to a greater extent in jejunal and FGCP transfectant membranes (>97% each) than in ileal membranes (44–48%).

mock transfected PC3 cells and exhibited a different optimal pH 4.5 with complete inhibition by *p*-hydroxymercuribenzoate. The kinetic characteristics of FGCP activity were compared in membranes from FGCP and PSM transfectants and in purified pig jejunal brush borders. As shown in Fig. 3 (*right panel*) and summarized in Table II, K_m and V_{max} values were similar in all three samples and were consistent with the kinetic profile of purified pig jejunal brush-border FGCP (4).

Immunoblots—Fig. 4 compares the immunoreactivities of the monoclonal antibody Mab-3 (5) with purified native pig FGCP, with pig FGCP transfectant membranes before and after treatment with peptide-*N*-deglycosidase F, and with human PSM transfectant membranes. Mab-3 detected the native pig FGCP and the pig FGCP transfectant glycoprotein at the identical size of 120 kDa and detected the deglycosylated polypeptide at 84 kDa but did not react with the human PSM transfectant membranes or with mock transfected control membranes.

Northern Blots—The cDNA of pig FGCP showed a strong hybridization signal at 2.8 kb in pig duodenum and jejunum and a faint signal in pig kidney, while no signal was detected in pig liver or ileum (Fig. 5). A band of similar size was identified in RNA extracts from pig and human jejunal mucosa. A positive actin signal was present in all samples. Several bands of hybridization appeared in RNA samples from rat and human brain and the LNCaP prostate carcinoma cell line (Fig. 6). Bands of roughly equal intensity were observed in rat brain at approximately 3.9, 2.95, and 2.8 kb, while a predominant species of 2.8 kb was found in human brain and in the human LNCaP prostate cancer cell line.

DISCUSSION

The present study has achieved the original molecular characterization of FGCP from pig jejunal mucosa. The authenticity of the pig FGCP cDNA sequence and its specific functional expression was established by (*a*) the incorporation of two native peptide sequences into the deduced amino acid sequence (Fig. 1), (*b*) the reproduction of the activity profile and kinetics of native pig FGCP (2, 4) in FGCP transfectant membranes (Fig. 3), (*c*) the immunoblot identification of the FGCP transcript by monoclonal antibody to native pig FGCP at the identical 120-kDa molecular size of the purified native enzyme (Ref. 5; Fig. 4) and identification of the deglycosylated polypeptide at the 84-kDa molecular size predicted by the amino acid sequence (Fig. 1), and (*d*) the identification of FGCP transcripts at 2.8 kb in pig jejunal mucosa and their absence in pig ileal mucosa (Fig. 5), consistent with the established intestinal distribution of the activity and immunoreactivity of the native enzyme (5). The additional presence of similar FGCP transcripts in pig and human jejunal mucosa (Fig. 5) suggests that the same gene expresses FGCP in human and pig jejunal brush-border membranes (2, 5).

The present experiments complete a circle of evidence for extensive molecular homologies among pig FGCP, human PSM, and rat NAALADase. The findings of 83–91% amino acid sequence identities between pig FGCP and each of the other sequences (Fig. 1; Table I) is in keeping with prior reports on the extensive amino acid identities between human PSM and rat NAALADase (6–9, 11) and is consistent with the concept that all three proteins represent species-specific homologues of the same gene. While the amino acid sequence of each protein predicts a polypeptide molecular size of 84 kDa (Fig. 1; Refs. 6–8), the presence of 12 glycosylation sites accounts for the greater 120-kDa molecular size of native (5) or transfectant FGCP (Fig. 4) compared with the reported molecular sizes of 100 kDa for PSM with 10 glycosylation sites (6) and of 94 kDa for NAALADase with nine glycosylation sites (7, 8, 34). While the epitope for our monoclonal antibody to native pig FGCP is unknown, incomplete amino acid sequence identities and differences in glycosylation between pig FGCP and human PSM could account for the lack of antibody cross-reactivity with PSM in transfectant membranes (Fig. 4). Prior findings of NAALADase transcripts at 2.8 kb in rat kidney (7, 8) are extended by the detection of a weak FGCP hybridization signal at 2.8 kb in pig kidney poly(A$^+$) RNA (Fig. 5), while the prior findings of PSM-like transcripts and immunoreactivity in human small intestine (35–37) are complemented by the detection of the FGCP hybridization signal at 2.8 kb in pig duodenal and jejunal poly(A$^+$) RNA and in human jejunal RNA (Fig. 5). The tissue distribution and predominant size of FGCP-like transcripts in rat and human brain and LNCaP cells (Fig. 6) is similar to other descriptions of the distribution and sizes of PSM and NAALADase transcripts in these tissues (6–9, 38). The previous finding of NAALADase activity in membranes of LNCaP cells and PSM transfectants (9) is complemented by finding NAALADase activity in pig jejunal brush-border membranes and in FGCP transfectant membranes (Fig. 2). The

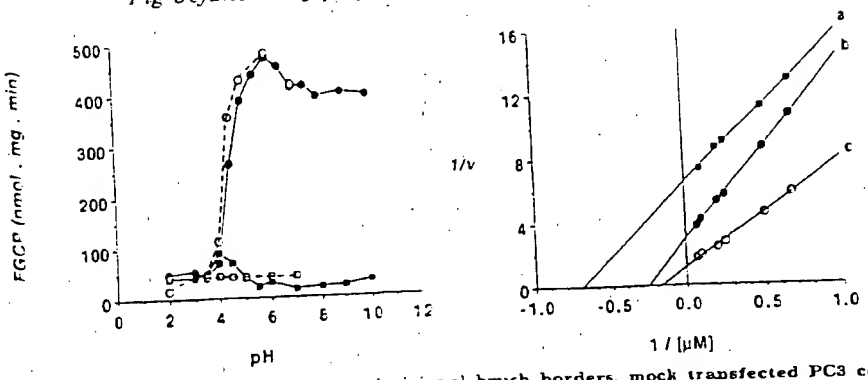

FIG. 3. **Folate hydrolysis by membranes from native pig jejunal brush borders, mock transfected PC3 cells, and PC3 cells transfected with the cDNA of FGCP or PSM.** Reaction mixtures consisted of 12 μM substrate folyl-γ-Glu-γ-[^{14}C]Glu in 33 mM 3,3-dimethylglutarate buffer containing 0.1 mM zinc acetate and 0.67 M NaCl in the final concentration. *Left panel*, Effect of varied buffer pH on folate hydrolysis by membranes from mock transfected and FGCP-transfected PC3 cells. FGCP activity was optimal in FGCP transfectant membranes at pH 6.0 (*closed circles*), in contrast to lesser folate hydrolysis in mock transfected PC3 cell membranes at optimal pH 4.0 (*closed boxes*). The addition of 0.5 mM *p*-hydroxymercuribenzoate in the final concentration had no effect on FGCP activity in FGCP transfectant membranes (*open circles*) but resulted in complete inhibition of folate hydrolysis in control PC3 cell membranes (*open boxes*). The FGCP activity profile of membranes of PSM transfectants was identical to that of FGCP transfectants (not shown). *Right panel*, kinetics of FGCP activity in membranes from pig jejunal brush borders and PC3 cells transfected with the cDNA of FGCP or PSM. Lineweaver-Burk plots of kinetics at pH 6.5 over a range of folyl-γ-Glu-γ-[^{14}C]Glu substrate concentrations show near identity among the membranes. *a*, PSM transfectant membranes; *b*, purified native pig jejunal brush-border membranes; *c*, FGCP transfectant membranes. K_m and V_{max} kinetic values are compared in Table II.

TABLE II

FGCP kinetics in native pig and transfectant cell membranes

A summary of activity constants (K_m) and maximal activities (V_{max}) of FGCP in membranes from purified pig jejunal brush borders, PC3 cells transfected with the cDNA of FGCP or PSM, and previously reported purified native pig jejunal FGCP (4). Kinetic data were obtained from studies that used a range of concentrations of substrate folyl-γ-Glu-γ-[^{14}C]Glu at pH 6.5 and conditions as described under "Experimental Procedures," followed by Lineweaver-Burk analysis of the results as shown in Fig. 5.

Source	K_m	V_{max}
	μM	nmol · mg · min
Pig jejunal brush border membrane	3.9	338
FGCP transfectant membrane	5.8	858
PSM transfectant membrane	1.4	152
Purified pig jejunal FGCP (4)	1.7	540

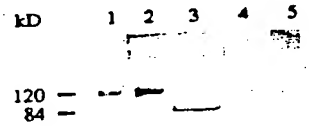

FIG. 4. **Immunoblots showing the reaction of monoclonal antibody to native pig FGCP (5) to transfectant membrane proteins.** Seven μg of solubilized membrane protein was added to each lane. An identical protein band was identified at 120 kDa in purified native pig FGCP (*lane 1*) and in membranes from the FGCP transfectant (*lane 2*), while the deglycosylated FGCP polypeptide appeared at 84 kDa (*lane 3*). Protein bands were absent from membranes of PSM transfectants (*lane 4*) and mock transfected PC3 cells (*lane 5*).

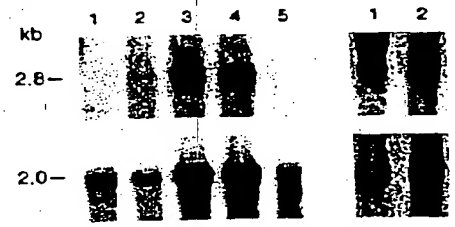

FIG. 5. **Northern hybridization of ^{32}P-labeled pig FGCP cDNA and human β-actin to pig and human tissues.** *Left panel*, a band of hybridization at 2.8 kb was prominent in poly(A$^+$) RNA from pig duodenal and jejunal mucosa (*lanes 3* and *4*), present in kidney (*lane 2*), and absent from liver (*lane 1*) and ileal mucosa (*lane 5*). *Right panel*, bands of hybridization of similar intensities were found at 2.8 kb in total RNA from pig (*lane 1*) and human jejunal mucosa (*lane 2*). Control hybridization to actin is shown at 2.0 kb.

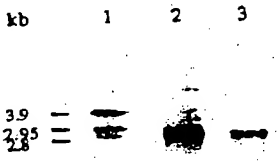

FIG. 6. **Northern hybridization of ^{32}P-labeled pig FGCP cDNA to brain and prostate RNAs.** Samples contained different amounts of total RNA in rat brain (10 mg) and LNCaP cells (5 mg) and poly(A$^+$) RNA in human brain (2 mg). A longer exposure was required to develop the signal from rat brain. Bands of hybridization were observed in rat brain RNA at 3.9, 2.95, and 2.8 kb (*lane 1*). A predominant hybridization signal appeared at 2.8 kb in LNCaP cell RNA (*lane 2*) and in human brain poly(A$^+$) RNA (*lane 3*).

observation that membranes of LNCaP cells or PSM transfectants were capable of progressive hydrolysis of folylpoly-γ-glutamates (10) is confirmed and extended by finding nearly identical kinetic properties of purified native FGCP in FGCP or PSM transfectant membranes (Fig. 3; Table II).

A recent analysis classified human prostate PSM and rat brain NAALADase as GCP II, a single type II glycoprotein member of the M28 family of peptidases (11) (EC 3.4.17.21). The extensive amino acid identities, common structural motifs, and conservation of the identical five co-catalytic zinc-binding amino acids and four putative substrate binding basic amino acids suggest that FGCP derives from the pig homologue of the GCP II gene (Fig. 1). GCP II and two prototypical bacterial aminopeptidases *V. proteolyticus* (27) and *S. griseus* (28) are members of the M28 peptidase family by virtue of homologous catalytic domains, which appear to bind two co-catalytic zinc

atoms (11, 39). The three-dimensional structural analysis of *V. proteolyticus* aminopeptidase suggested the location of a substrate specificity pocket, which is composed of basic amino acids in PSM and NAALADase (11, 27). The loci of the human PSM gene and a second similar sequence have been found on human chromosome 11 (40, 41). Others recently identified another type II ileal brush-border membrane protein, I100, that shares 60 and 59% sequence identities with rat NAALADase and human PSM (29), of which the human homologue might comprise the second locus on chromosome 11. I100 exhibits activity similar to human dipeptidyl peptidase IV, another peptidase associated with the apical brush border of intestinal epithelial cells (29, 30). These relationships prompted our evaluation of potential structural similarities among FGCP, I100, and dipeptidyl peptidase IV. The conservation of all five zinc-binding residues suggests that FGCP and I100 share the same catalytic mechanism. On the other hand, an alternative potential serine carboxypeptidase mechanism (29) is suggested by conservation of Ser^{632} in all three sequences.

While pig FGCP, rat NAALADase, and human PSM may represent different species-specific expressions of same GCP II gene, their functions appear to differ according to the tissue in which the gene is expressed. Thus, GCP II may function as FGCP in the jejunum by cleaving γ-linked glutamyl residues sequentially from dietary folylpoly-γ-glutamates prior to the intestinal transport of folic acid (1, 2, 4, 5) and as NAALADase in the brain to release α-linked glutamate from NAAG to regulate subsequent neurotransmission (8, 9). These different functions may reflect tissue differences in available substrate, since NAAG is concentrated at neuronal synapses (8), while folylpoly-γ-glutamates are concentrated as dietary components at the brush-border surface of the proximal small intestine (1).

The present study offers molecular clarity to the mechanism of folate absorption at the intestinal brush-border membrane. Our original studies identified an initial stage of jejunal hydrolysis of dietary folylpoly-γ-glutamates that precedes the intestinal uptake of the folic acid product (1). We identified and characterized FGCP as a zinc-dependent exopeptidase that is active at a neutral pH optimum in human and pig jejunal brush-border membrane fractions (2, 4) and that was localized in the pig to the jejunal brush-border membrane and was excluded from the ileal brush-border membrane by the monoclonal antibody Mab-3 to the purified enzyme (5). These observations are extended by the present molecular characterization of FGCP as a type II protein of the M28 peptidase family with a zinc-binding motif, for which the transcripts are expressed in proximal but not distal pig small intestine (Fig. 5). The finding of a different activity profile of folate hydrolysis by mock transfected PC3 cells including an acid pH optimum and complete *p*-hydroxymercuribenzoate inhibition (Fig. 3) is consistent with our prior definition of the characteristics of a separate lysosomal endopeptidase that provides intracellular folate hydrolysis in human and pig jejunal mucosa (3, 4). The recently described PSM′ splice variant (42) cannot provide the separate profile of folate hydrolysis found in mock transfected PC3 cells (Fig. 3), since no genetically similar species is expressed in native PC3 cells (6, 9). Alternatively, the second folate hydrolyzing activity in mock translated PC3 cell membranes (Fig. 3) and in the lysosomal fraction of jejunal mucosa (3) may be attributed to the recently described and genetically dissimilar glutamate hydrolase (EC 3.4.19.9) (Table I; Ref. 31).

The present studies provide a molecular framework for future studies on the regulation of FGCP by conditions known to affect intestinal folate absorption and on the relationship of FGCP to RFC and FBP, two proteins involved in membrane transport of monoglutamyl folates (Table I). The cDNA sequences of mouse and human RFC have been defined, and its intestinal transcription and functional capability for transport of monoglutamyl folate in cell transfectants has been proven (32, 43, 44). The alternate receptor FBP has been characterized at the molecular level in pig liver, but its transcripts and activity are absent from the jejunum (33).[2] The present study shows that FGCP is genetically distinct from both RFC and FBP, since their amino acid sequences are minimally represented in FGCP (Table I). In summary, the available data indicate that the intestinal absorption of dietary folylpoly-γ-glutamates is achieved by a two-step process of progressive hydrolysis of γ-linked glutamyl residues by FGCP at the jejunal brush-border membrane, releasing folic acid and other monoglutamyl folate derivatives for subsequent membrane transport by genetically distinct RFC. The integration of folate hydrolysis by jejunal FGCP and folic acid transport by intestinal RFC in the overall process of folate absorption has yet to be defined. These studies are now feasible due to the molecular identification of FGCP.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 261, No. 29, Issue of October 15, pp. 13551-13558, 1986
Printed in U.S.A.

Intracellular Pteroylpolyglutamate Hydrolase from Human Jejunal Mucosa

ISOLATION AND CHARACTERIZATION*

(Received for publication, March 17, 1986)

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Human jejunal intracellular pteroylpolyglutamate hydrolase was purified 30-fold from intestinal mucosa. The apparent molecular weight of the enzyme was 75,000 by Sephadex G-200 gel filtration, and the isoelectric point was at pH 8.0. The enzyme was maximally active at pH 4.5 and was unstable at increasing temperatures. Intracellular pteroylpolyglutamate hydrolase cleaved both terminal and internal γ-glutamate linkages. In contrast, brush-border pteroylpolyglutamate hydrolase catalyzed the hydrolysis of only terminal γ-glutamate linkages. The intracellular enzyme showed greatest affinity for the complete folic acid molecule with longer glutamate chains. Subcellular fractionation studies showed the intracellular enzyme was localized in lysosomes. These data show that the properties of human jejunal intracellular pteroylpolyglutamate hydrolase are distinct from those of the brush-border enzyme but are similar to the properties of intracellular pteroylpolyglutamate hydrolase described in other tissues.

Pteroylpolyglutamate hydrolases catalyze the hydrolysis of pteroylpolyglutamates to derivatives with shorter glutamate chains. These enzymes have been described in the intestinal mucosa of several different species. Only the intracellular form of pteroylpolyglutamate hydrolase has been found in the intestinal mucosa of most animals, whereas two forms of the enzyme have been identified in human and pig intestinal mucosa. The first is associated with the brush-border membrane, and the second is soluble and in the intracellular fraction (1). We recently described the purification and properties of human brush-border pteroylpolyglutamate hydrolase and showed that this enzyme is involved in the digestion of pteroylpolyglutamate, the predominant form of dietary folate (2). Relatively little is known, however, about the properties of human intestinal intracellular pteroylpolyglutamate hydrolase and its possible role in folate metabolism. Our present objectives were to isolate and characterize intracellular pteroylpolyglutamate hydrolase from human intestinal mucosa and to compare its properties with those of the human intestinal brush-border enzyme. These data show distinct properties for each hydrolase and suggest that intracellular pteroylpolyglutamate hydrolase may play a role in cellular folate metabolism that is unrelated to the digestion of dietary folates.

* This work was supported by an institutional award from Bristol-Myers Co. and Grants AM18330 and AM35747 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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EXPERIMENTAL PROCEDURES[1]

RESULTS

Physical Properties—Intracellular pteroylpolyglutamate hydrolase was purified 30-fold (Table I). The apparent molecular weight was estimated by gel filtration to be 75,000. The isoelectric point was at pH 8.0. Maximal activity of the enzyme occurred at pH 4.5 (Fig. 1*A*) and at 65 °C (Fig. 1*B*). The enzyme was unstable at 37 °C in pH 4.5 assay buffer alone (Fig. 1C). However, the linearity of the product *versus* time curve for up to 45 min (Fig. 1*D*) indicated a protective effect of the substrate at 37 °C and ensured the validity of the enzyme assays. The activity of intracellular pteroylpolyglutamate hydrolase was unaffected by dialysis against 1 mM EDTA, and the addition of 100 μM zinc acetate to the reaction mixture resulted in 15% inhibition.

Affinity for Substrate—The K_m for $PteGlu_3$,[2] determined from a Lineweaver-Burk plot, was 1.2 μM. Fig. 2 shows reciprocal plots of $PteGlu_3$ hydrolysis in the presence of varied concentrations of $PteGlu_7$. This compound was a competitive inhibitor of the reaction, with a K_i of 0.09 μM obtained from a replot of the x intercepts. $PteGlu_2$ also showed similar inhibition characteristics but had lower affinity for the enzyme, with a K_i of 1.2 μM (data not shown). The effects of various $PteGlu_n$ moieties on the activity of the enzyme is shown in Table II. Complete inhibition of $PteGlu_3$ hydrolysis was observed with $PteGlu_2$ and $PteGlu_7$ at 0.1 mM. Both PteGlu and $H_4PteGlu$ at 0.1 mM caused a 15% inhibition and at 1.0 mM, a 60% inhibition. At 1 mM, pterine and γ-diglutamate showed 50 and 30% inhibition, respectively. There was a slight inhibition by 1 mM *p*-aminobenzoylglutamic acid and no inhibition by 1 mM glutamic acid, α-diglutamic acid, or α-triglutamic acid (Table II).

[1] Portions of this paper (including "Experimental Procedures" and Figs. 1-5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-0536, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: $PteGlu_3$, pteroyltriglutamic acid; $PteGlu_7$, pteroylheptaglutamic acid; PteGlu, folic acid; $PteGlu_2$, pteroyldiglutamic acid; $H_4PteGlu$, tetrahydropteroylglutamic acid; $PteGlu_n$, pteroylpolyglutamic acid; $PteGlu_2$[^{14}C]glu, pteroyldiglutamyl[^{14}C]glutamic acid; $PteGlu_2$[^{14}C]gluLeu$_2$, pteroyldiglutamyl[^{14}C]glutamyldileucine; HPLC, high pressure liquid chromatography.

Applicants: Ron S. Israeli, et al.
Serial No.: 08/470,735
Filed: June 6, 1995
Exhibit 4

TABLE I

Partial purification of intracellular pteroylpolyglutamate hydrolase

Procedure	Total protein	Protein	Total activity	Specific activity	Recovery	Purification factor
	mg	*mg/ml*	*milliunits*	*milliunits/mg*	%	*-fold*
10% homogenate*	4850	17.2	733.2	0.15	100	1
30,000 × *g* supernatant	1849	6.6	559	0.3	76	2
pH precipitation	940	4.7	480	0.51	65	3.4
$(NH_4)_2SO_4$ precipitation	392	14.0	210	0.54	30	3.6
Isoelectric focusing	24.1	1.46	100	4.14	14	27.6

* Homogenate was made from 20 g of tissue.

TABLE II

Effect of different moieties of $PteGlu_n$ on $PteGlu_3$ hydrolysis

Compound	% of control activity	
	0.1 mM	1 mM
PteGlu	85	36
$H_4PteGlu$	85	40
$PteGlu_2$	0	0
$PteGlu_1$	0	0
p-Aminobenzoylglutamic acid	92	85
Pterine	96	47
Glutamic acid	92	97
γ-Glutamylglutamic acid	100	70
α-Glutamylglutamic acid	100	100
α-Glutamylglutamylglutamic acid	100	100

Mechanism of Hydrolysis—As shown in Fig. 3, the labeled products resulting from hydrolysis of $PteGlu_2$[^{14}C]Glu were equal amounts of [^{14}C]glutamic acid and γ-glutamyl[^{14}C]glutamic acid. Timed incubations of the enzyme with $PteGlu_7$ showed the rapid appearance of folic acid (PteGlu), with minimal accumulation of intermediate products (Fig. 4). Incubation of the enzyme with $PteGlu_2$[^{14}C]$GluLeu_2$ resulted in a release of radioactivity that corresponded to 5% of the hydrolysis rate when using $PteGlu_3$ as substrate.

Subcellular Location—Using fresh tissue, intracellular pteroylpolyglutamate hydrolase was localized in the fractions enriched with mitochondria and lysosomes (Fig. 5*A*). Freezing and thawing of the tissue resulted in a similar redistribution of the lysosomal marker enzyme and intracellular pteroylpolyglutamate hydrolase (Fig. 5*B*). More than 80% of both the lysosomal marker *N*-acetylglucosaminidase and pteroylpolyglutamate hydrolase appeared in the soluble fraction. Other marker enzymes showed no changes when compared to fresh tissue.

DISCUSSION

The absorption of dietary folate is attributed in part to the activity of specific pteroylpolyglutamte hydrolases located in the intestinal mucosa. To understand the mechanisms involved in absorption of dietary folate, we have focused our studies on two pteroylpolyglutamate hydrolases in human intestinal mucosa. Recently, we reported on the purification and properties of the brush-border enzyme (2). In the present study, we have examined the properties of the intracellular pteroylpolyglutamate hydrolase to understand the possible relationship of the two enzymes in folate digestion and metabolism.

As shown in Table I, a 30-fold purification of intracellular pteroylpolyglutamate hydrolase was achieved. The enzyme has an apparent molecular weight of 75,000, optimal activity at pH 4.5, a pI of 8.0, and instability at increasing temperature. The inhibition of $PteGlu_3$ hydrolysis by $PteGlu_2$ (K_i = 1.2 μM) and $PteGlu_7$ (K_i = 0.09 μM) showed competitive inhibition patterns with Lineweaver-Burk plots, indicative of greater affinity for longer chain pteroylpolyglutamates. Inhibition of $PteGlu_3$ hydrolysis by $PteGlu_n$, and to a lesser extent by other folate derivatives, and the lack of inhibition by α-glutamates or other moieties suggest that the enzyme requires both the complete folic acid moiety and γ-glutamate linkage for activity. The enzyme is capable of cleaving both terminal and internal γ-peptide bonds since incubation of intracellular pteroylpolyglutamate hydrolase with $PteGlu_2$[^{14}C]Glu resulted in the release of both ^{14}C-labeled glutamic acid and ^{14}C-labeled diglutamic acid. The release of radioactivity when the enzyme was incubated with $PteGlu_2$[^{14}C]$GluLeu_2$ and the minimal accumulation of the intermediate product with $PteGlu_7$ incubation support this conclusion. Subcellular fractionation studies using differential centrifugation demonstrated that the intracellular pteroylpolyglutamate hydrolase is located in the lysosomes.

TABLE III

Comparison of intracellular pteroylpolyglutamate hydrolase and brush-border pteroylpolyglutamate hydrolase

Property	Intracellular	Brush border
Apparent M_r	75,000	700,000
pH optimum	4.5	6.5
pI	8.0	7.2
Reducing agent requirement	Yes	No
Temperature stability	No	Yes
Metal requirement	No	Yes (Zn^{2+}, Co^{2+})
K_m for $PteGlu_3$ (μM)	1.2	0.6
K_i for $PteGlu_7$ (μM)	0.09	0.6
Mechanism of hydrolysis	Cleaves both terminal and internal linkages	Exopeptidase
Final product	PteGlu	PteGlu
Localization	Lysosome	Brush border

Comparisons of the properties of human intracellular and brush-border pteroylpolyglutamate hydrolase indicate that they are distinct enzymes (Table III). The differences between these two enzymes include molecular weight, optimum pH, temperature stability, and requirement for metal ions and a reducing agent. Both enzymes showed similar K_m values for $PteGlu_3$ and greatest affinity when both the folic acid moiety and the γ-glutamate bond were present. However, intracellular pteroylpolyglutamate hydrolase had greater affinity for folates with longer glutamate chains, whereas the brush-border enzyme had no preference for the number of glutamate residues. Whereas intracellular pteroylpolyglutamate hydrolase is capable of cleaving both internal and terminal γ-glutamate linkages, the brush-border enzyme is an exopeptidase (2).

Comparisons of human intestinal intracellular pteroylpolyglutamate hydrolase with pteroylpolyglutamate hydrolases from other mammalian tissues reveal similarities and differences. Similar properties of pteroylpolyglutamate hydrolases have been described in human liver (16), bovine liver (17), rat liver (18), hog kidney (19), guinea pig intestine (20), and rat intestine (21). In each site, the enzyme had an acidic pH optimum and was demonstrated to be lysosomal in human

liver, rat liver, and guinea pig intestine. The ability to cleave internal γ-glutamate bonds was observed in studies of pteroylpolyglutamate hydrolase isolated from bovine liver and rat intestine, whereas exopeptidase activity was observed in human liver and hog kidney. Affinity toward longer glutamate chains was observed in both bovine liver and rat intestine. Furthermore, sensitivity to sulfhydryl agents and the protective effect of reducing agents were properties of pteroylpolyglutamate hydrolase from human liver, bovine liver, and hog kidney, which suggests involvement of SH groups in activity.

The role of intracellular pteroylpolyglutamate hydrolase in the human intestinal mucosa is obscure. A possible involvement of the intracellular pteroylpolyglutamate hydrolase in the absorption of dietary folate is not excluded but would require transport of all or part of the pteroylpolyglutamates into the cell prior to hydrolysis. Intracellular pteroylpolyglutamate hydrolase may function in regulating the levels of pteroylpolyglutamates within the enterocyte since others have demonstrated the capability for synthesis of these forms of the vitamin by intestinal mucosa (22). The similarities between human intestinal intracellular pteroylpolyglutamate hydrolase and the intracellular enzyme from other mammalian tissues imply that these enzymes have similar roles in cellular folate metabolism. Furthermore, pteroylpolyglutamate is not only the preferred coenzyme for many folate-dependent enzymes in single carbon transfer reactions but also has been found to be an effective inhibitor of a number of enzymes, including thymidylate synthetase and methylene-H_4PteGlu reductase (23, 24). Others observed increased glutamylation of folate in hepatoma cells in the presence of insulin or dexamethasone (25). These observations suggest that a fairly complex regulation of pteroylpolyglutamate levels exists in the cell and implies that jejunal mucosal intracellular pteroylpolyglutamate hydrolase may play a significant physiological role in cellular metabolism.

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SUPPLEMENTAL MATERIAL TO
INTRACELLULAR PTEROYLPOLYGLUTAMATE HYDROLASE FROM HUMAN JEJUNAL MUCOSA:
ISOLATION AND CHARACTERIZATION

T.T.Y. Wang, C.J. Chandler, C.H. Halsted

EXPERIMENTAL PROCEDURES

Source of Tissue. Human jejunal mucosa was obtained from patients undergoing elective jejunoileal bypass for obesity or revision of this operation. The amount of jejunum resected at each operation did not exceed that required for the surgical procedure. After the jejunal segment was washed in ice-cold saline in the operating room, the mucosa was rapidly dissected, wrapped in parafilm, and frozen with dry ice. Each sample was stored at -70° C until further use.

Chemicals. [^{3}H]PteGlu was purchased from Amersham. PteGlu$_2$[^{14}C]Glu, PteGlu$_3$, PteGlu$_7$, and PteGlu$_2$[^{14}C]GluLeu$_2$ were synthesized by the solid phase method (3) and provided by Dr. Carlos Krumdieck, University of Alabama. All other chemicals were obtained from commercial sources.

Assays. The activity of intracellular pteroylpolyglutamate hydrolase was measured by a modification of the charcoal adsorption method (4). The standard reaction mixture consisted of 13 μM PteGlu$_2$[^{14}C]Glu, 66 μM glutamic acid, 33 mM sodium acetate buffer at pH 4.5, and enzyme in a final volume of 0.75 ml. After incubation at 37° C for 15 min, reactions were terminated by the addition of 0.25 ml of 10% trichloroacetic acid. Unreacted substrate was adsorbed with 0.5 ml of 3% charcoal in 0.1 M acetic acid. After centrifugation, the radioactivity liberated by the enzyme was quantitated by liquid scintillation counting. Under these conditions, less than 10% of the substrate was hydrolysed during the reaction period. To differentiate the activities of the two human jejunal mucosal pteroylpolyglutamate hydrolases in the initial mucosal homogenate, the assay was carried out in the presence and absence of 0.17 mM p-hydroxymercuribenzoate. At this concentration, p-hydroxymercuribenzoate completely inhibits intracellular pteroylpolyglutamate hydrolase and has no effect on the brush border enzyme (5). The initial activity of intracellular hydrolase is expressed as the difference of the activities measured with and without the inhibitor.

After the initial centrifugation all enzyme activity was inhibited by p-hydroxymercuribenzoate and was omitted in assays of the subsequent preparations. Protein concentration was measured by the Bradford method (6). The activity of intracellular pteroylpolyglutamate hydrolase was expressed as milliUnits per mg of protein, where one milliUnit is defined as one nmole of substrate hydrolysed per min.

PURIFICATION

Homogenization. A 10% homogenate (w/v) was prepared from minced mucosa in 50 mM Tris-Cl buffer, pH 7.0, using a Brinkman polytron homogenizer, followed by vacuum filtration through cheesecloth. A supernatant fraction containing intracellular pteroylpolyglutamate hydrolase was obtained after centrifugation at 30,000 x g for 15 min, and 2-mercaptoethanol was added to the supernate to a final concentration of 10 mM. Since intracellular pteroylpolyglutamate hydrolase was sensitive to oxidation, preservation of its activity required a reducing agent.

pH Precipitation. The supernate was adjusted to pH 4.5 with 12 M HCl, followed by centrifugation at 30,000 x g for 15 min. The pellets were discarded, and the pH of the supernate was adjusted to pH 7.0 with 10 M NaOH.

Ammonium Sulfate Precipitation. The supernate was concentrated by ammonium sulfate precipitation. A pellet from 40% to 80% saturation was obtained and dissolved in a minimal volume of 50 mM sodium acetate buffer containing 10 mM 2-mercaptoethanol, at pH 7.0, and subsequently dialysed against the same buffer (100 x sample volume, 3 changes, 8 hr) to remove ammonium sulfate.

Isoelectric Focusing. The concentrated sample was applied to an LKB isoelectric focusing column (LKB 8101, 110 ml) and underwent isoelectric focusing for 48 hr in a 0-40% sucrose gradient with 2% ampholyte (pH 4.5-9.0) at a constant voltage of 600 V. Fractions containing enzyme were pooled and dialysed against 50 x volume of 50 mM sodium acetate buffer containing 10 mM 2-mercaptoethanol, 10% sucrose at pH 7.0, with 3 changes for 8 hr to remove ampholyte. The sample was stored at 4° C and subsequently used for characterization studies.

CHARACTERIZATION

Molecular Weight Determination. Molecular weight was determined by gel filtration in Sephadex G-200 (2 x 100 cm) with blue dextran (void volume), catalase (232,000), aldolase (158,000), bovine serum albumin (67,000), and cytochrome c (13,000) as markers.

Effects of pH and Temperature. The pH and temperature dependence of pteroylpolyglutamate hydrolase activity and stability were determined over a range of conditions as detailed in Fig. 1.

Effect of Metal Ions. Standard assays for pteroylpolyglutamate hydrolase were performed after dialysis of the enzyme against 1 mM EDTA. The effect of zinc was also examined by adding zinc acetate (100 μM) to the reaction mixture using nondialysed enzyme.

Affinity for Substrate. The kinetic constants of intracellular pteroylpolyglutamate hydrolase for PteGlu$_2$[^{14}C]Glu were determined by measuring the initial reaction velocities at varied substrate concentrations. The effects of glutamate chain length on enzyme activity were studied by measuring the initial velocities of PteGlu$_2$[^{14}C]Glu hydrolysis in the presence of different fixed concentrations of PteGlu$_3$ (0-5 μM) or PteGlu$_7$ (0-0.5 μM). Substrate specificity was determined by examining the effects of various moieties of PteGlu$_n$ on activity. The reaction was measured using PteGlu$_2$[^{14}C]Glu with the addition of 0.1 mM or 1.0 mM of PteGlu, H$_4$PteGlu, Glu, γ-Glu$_2$, α-Glu$_2$, α-Glu$_3$, N-p-aminobenzoylglutamate, or pterine.

Mechanism of Hydrolysis. The mechanism of hydrolysis of intracellular pteroylpolyglutamate hydrolase was studied by chromatographic identification of the products of PteGlu$_2$[^{14}C]Glu using an AG1-X8 ion exchange column (Bio-Rad) and of the products of PteGlu$_7$ hydrolysis using a modification of the HPLC method with detection at 280 nm as described by Shane et al. (7). To confirm the ability of intracellular pteroylpolyglutamate hydrolase to cleave internal γ-glutamate linkages, activity was measured using the standard assay, substituting PteGlu$_2$[^{14}C]GluLeu$_2$ (50 μM) as the substrate. Release of radioactive product from this compound would require the hydrolysis of internal γ-glutamate linkages.

Subcellular Localization. A 15% homogenate (w/v) was made from either fresh or previously frozen tissue in 5.45% sorbitol, 1 mM phosphate buffer, pH 7.0. After filtration through cheesecloth, subcellular fractions were obtained by differential centrifugation following the method described by Mejia et al. (8). Marker enzymes included lactate dehydrogenase for cytosol (9), sucrase and brush border pteroylpolyglutamate hydrolase for brush borders (3,10), N-acetylglucosaminidase for lysosomes (11), Na$^+$-K$^+$ ATPase for basolateral membranes (12), aryl esterase for endoplasmic reticulum (13), and succinate-INT reductase and glutamate dehydrogenase for mitochondria (14,15). The distribution patterns of intracellular pteroylpolyglutamate hydrolase were compared with marker enzymes in fresh and frozen tissue.

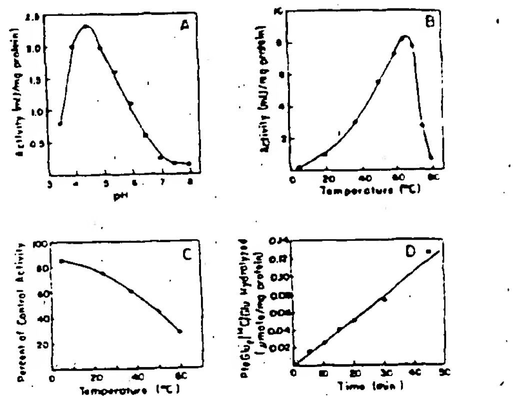


Figure 1. Dependence of intracellular pteroylpolyglutamate hydrolase on pH and temperature.

A. pH-dependent activity profile of intracellular pteroylpolyglutamate hydrolase. Enzyme was assayed in the standard reaction mixture using 33 mM 3,3-dimethylglutarate at varied pH for 15 min.

B. Temperature-dependent activity profile of intracellular pteroylpolyglutamate hydrolase. Enzyme was assayed in the standard reaction mixture at varied temperatures for 15 min.

C. Temperature stability of intracellular pteroylpolyglutamate hydrolase. Enzyme was preincubated at various temperatures in the reaction mixture without substrate for 20 min. Bovine serum albumin (0.5 mg/ml) and pepstatin (0.2 μM) were added to prevent proteolysis. After cooling on ice, enzyme activity was assayed at 37° C as described under Experimental Procedures.

D. Product production over time. Standard reactions were performed for 5, 10, 15, 20, 30, and 45 min.

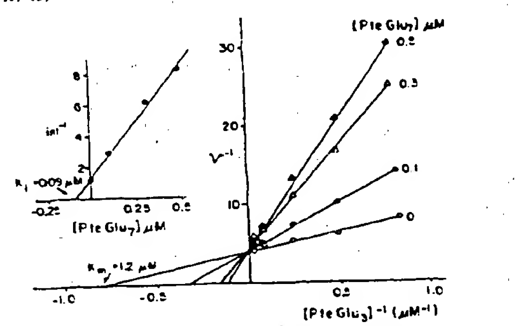


Figure 2. Inhibition of PteGlu$_3$ hydrolysis by PteGlu$_7$. The initial velocities at varied concentrations of PteGlu$_2$[^{14}C]Glu in the presence of different concentrations of PteGlu$_7$ were determined in standard reaction mixtures. The reactions were started by the addition of enzyme. Data were plotted as initial velocity^{-1} versus [PteGlu$_3$]$^{-1}$. Y-int is the y-intercept of the primary plot.

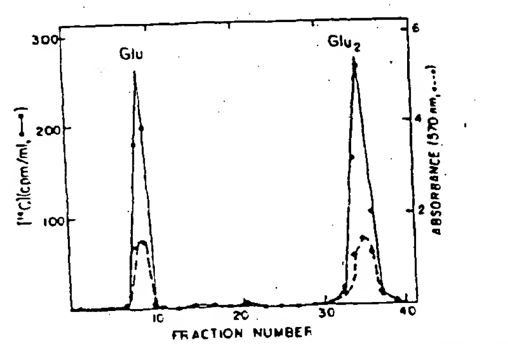


Figure 3. Glutamate reaction products of intracellular pteroylpolyglutamate hydrolase. Enzyme was incubated in the standard reaction mixture for 1 hr. The reaction was stopped with 3 ml of cold buffer containing 5 mM phosphate (pH 7.0), 0.1 mM HgCl$_2$, and 10 μmoles of glutamic acid and 5 μmoles of γ-glutamylglutamate as marker. The sample was applied to a 1 x 30 cm Bio-Rad AG1-X8 column equilibrated with 5 mM phosphate buffer (pH 7.0). The column was eluted with a 0 to 0.3 M NaCl gradient (300 ml total volume), while collecting 5 ml fractions. The markers were detected using ninhydrin reagent and radioactivity determined by scintillation counting.

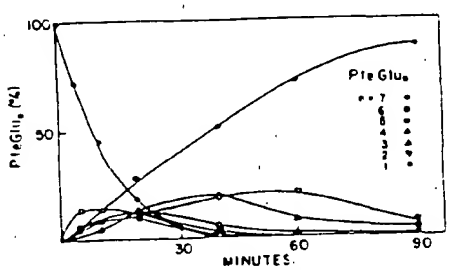
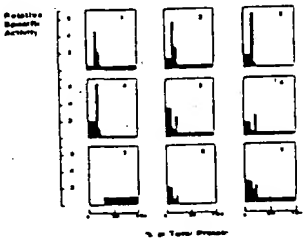


Figure 4. Pteroylglutamate reaction products of intracellular pteroylpolyglutamate hydrolase and PteGlu$_7$. Enzyme was incubated in the standard reaction mixture with 32 μM PteGlu$_7$ as substrate. The reaction was started with 6.73 milliUnits of enzyme and incubated for 0, 5, 10, 20, 40, 60, and 90 min at 37° C. The reaction was terminated by placing the reaction tube in boiling water for 10 min and subsequently filtering it on a Millipore Swinnex-13 filtering apparatus. 150 μl of the filtrate was applied to a Whatman Partisil 10 SAX column for HPLC determination of products. The results are expressed as percent of total PteGlu$_n$.



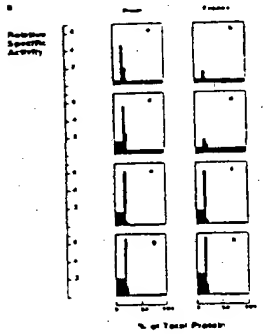


Figure 5. Distribution patterns of marker enzymes and intracellular pteroylpolyglutamate hydrolase.

A. The subcellular fractions obtained by starting with fresh tissue included A) 1,000 x g x 10 min, B) 8,000 x g x 10 min, C) 17,000 x g x 10 min, and D) 17,000 x g x 10 min supernate. Marker enzymes, as described in Experimental Procedures, were assayed for each fraction. Activity is expressed as relative specific activity (specific activity of the fraction divided by the specific activity of the original homogenate). 1. intracellular pteroylpolyglutamate hydrolase, 2. N-acetylglucosaminidase, 3. succinate-IFT-reductase, 4. glutamate dehydrogenase, 5. brush border pteroylpolyglutamate hydrolase, 6. sucrase, 7. lactate dehydrogenase, 8. aryl esterase, 9. Fe^{++}-E ATPase.

B. Comparison of distribution patterns of lysosomal and mitochondrial marker enzymes in fresh and previously frozen and thawed tissue. Figures a and b are intracellular pteroylpolyglutamate hydrolase, c and d are N-acetylglucosaminidase, e and f are succinate-IFT reductase, and g and h are glutamate dehydrogenase.

(Translation) Mailed: March 2, 2004

NOTIFICATION OF REASONS FOR REJECTION

Patent Application No.: 511426/94

Examiner's Notice Date: February 20, 2004

Examiner: Eiji Tababori

This application is rejected on the grounds stated below. Any opinion about the rejection must be filed within THREE MONTHS of the mailing date hereof.

REASONS

1. The invention described in Claim 39 of the present application is directed to a diagnosing method for humans and therefore it is unpatentable under the main provision of Section 29(1) of the Patent Law, on the grounds that it does not appear to be industrially available.

2. The invention is unpatentable under Section 29 (1) (iii) of the Patent Law as being described in the following publication distributed in Japan or a foreign country prior to this application.

Remarks

Claims 23 and 24
Reference 1
Notes:

Reference 1 discloses a rat protein of 94 kDa and it is noted that this protein includes an antigenic fragment of a prostate-specific membrane antigen such as of the present application. (See Proc. Natl. Acad. Sci. USA., Vol. 93, No.2 (1996) pages 749 to 753 (Document 1), if necessary.)

Claims 23 and 24
Reference 2
Notes:

Reference 2 discloses a human protein of 75 kDa and it is noted that this protein includes an antigenic fragment of a prostate-specific membrane antigen such as of the present application. (See J. Biol. Chem., Vol. 273, No. 32 (1998) pages 20417 to 20424

(Document 2), if necessary.)

Claims 27 to 30 and 32 to 34
Reference 1
Notes:

Reference 1 discloses a polyclonal antibody to be bound to rat protein of 94 kDa and it is noted that the antibody is bound to an antigenic fragment of a prostate-specific membrane antigen such as of the present application. (See Proc. Natl. Acad. Sci. USA., Vol. 93, No.2 (1996) pages 749 to 753 (Document 1), if necessary.)

3. The invention is unpatentable under Section 29 (2) of the Patent Law, as being such that the invention could easily have been made by a person with ordinary skill in the art to which the invention pertains, on the basis of the invention described in the following publication(s) distributed in Japan or a foreign country prior to this application.

Claims 27 to 34
References 1 and 2
Notes:

It would be easily achievable for a person having ordinary skill in the art to obtain an antibody to be bound to a protein disclosed in Reference 1 or 2 by using the protein as an immunogen.

4. The application fails to satisfy the requirements under Section 36 (4) or (5) and (6) of the Patent Law, on the grounds that the specification and the drawing(s) are defective in the following respect(s).

(1) The recitation of Claim 1 is not definite as to the scope of peptide covered by the term "antigenic fragment". Thus, this claim is unclear with regard to the matters essential to the structure of the invention recited in the claim. This also case for the inventions recited in Claims 5, 6, 23 and 26.

(2) The recitation of Claim 2 is not definite as to the element i) "a fragment that is characterized by its antigenicity ..." and the scope of peptide included in the fragments recited in the elements h) to j). Thus, this claim is unclear with regard to the matters essential to the structure of the invention recited in the claim. This also case for the

invention recited in Claim 24.

(3) The recitation of Claim 6 is not clear as to under what condition it "hybridizes specifically to a nucleic acid", and as to the scope of DNA, which is a chemical substance. Thus, this claim is unclear with regard to the matters essential to the structure of the invention recited in the claim.

(4) Claim 6 recites "which hybridizes specifically to a nucleic acid according to any one of Claims 1 to 5"; however, the nucleic acids recited in Claims 1 to 5, contain a sequence that has a degeneration relationship with regard to a natural sequence, and therefore the recitation of this claim is technically not appropriate.

(5) Claim 13 recites an invention of the plasmid deposited under ATCC75294. The Detailed Description of the Invention describes that the plasmid was deposited in the ATCC on August 14, 1992. Nevertheless, a copy of the deposit certificate that certifies the deposit of the plasmid is not attached to the application. Therefore, it is not judged that the invention recited in this claim is described sufficiently in the Detailed Description of the Invention for a person having ordinary skill in the art to be able to easily carry out the invention.

(6) Claims 18 and 19 contains the expression "... which hybridizes specifically to a nucleic acid", which does not specifically describe of what nucleotide sequence the nucleic acid is made. Thus, the scope in which the nucleic acid is covered is not definite. Therefore, the claim is unclear with regard to the matters essential to the structure of the invention recited in the claim.

(7) Claim 32 recites an invention of a composition; however, the composition is not described definitely as to what components in combination it is made of, or the composition ratio is not described or the composition is not specified by its usage or characteristics. Therefore, this claim is unclear with regard to the matters essential to the structure of the invention recited in the claim. This also case for the inventions recited in Claims 33 to 36.

(8) Claim 36 does not indicate its pharmaceutical usage in terms of the pharmacological effect. Further, the end of this claim does not match with that of the claim from which Claim 36 is dependent, and therefore the recitation of this claim is not appropriate. (The pharmacological effect should be described in general pharmacological terms.)

(9) The invention of "use" recited in Claims 38, 39, 41 and 42, is not clear as to what category of patent it belongs to, that is, it is a "product", "method" or "method of producing the product".

The claims not mentioned in this Official Action are not rejected. If a new reason for rejection is noticed, a further Official Action will be issued.

LIST OF REFERENCES

1. J. Biol. Chem., Vol. 265, No. 34 (1990) pages 2197 to 21301

2. J. Biol. Chem., Vol. 261, No. 29 (1986) pages 13551 to 13555

If the applicant has any questions or wishes to have an interview, please contact the following Examiner: Eiji Takabori, the 3rd Division of Patent Examination (Biotechnology), Tel. 03-3581-1101, Extension 3447 to 3448